Volume 16 MAY-JUNE, 1951 Number 3

Food Research

An Official Publication of

The Institute of Food Technologists

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—NICOLAS APPERT.

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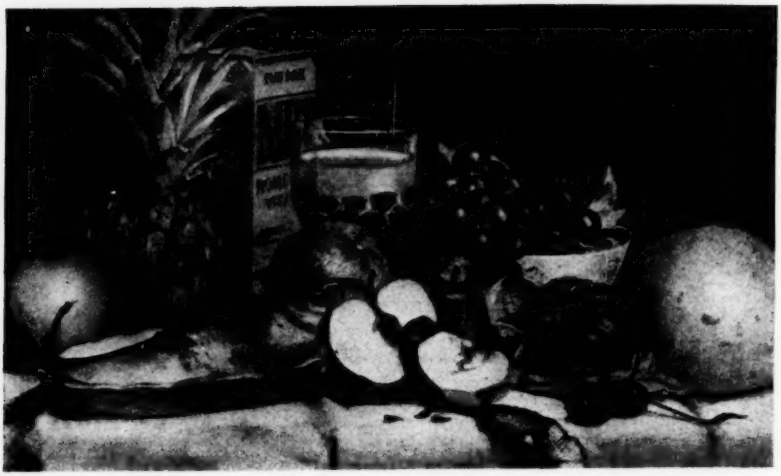

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ISOLATION AND CHARACTERIZATION OF PEACH TANNINS[a, b]

GESTUR JOHNSON, M. M. MAYER, AND D. K. JOHNSON
Chemistry Section, Colorado Agricultural Experiment Station, Fort Collins, Colorado

(Manuscript received May 29, 1950)

The polyphenolic or tannin-like substances present in peaches and other fruits are important substances from the standpoint of astringent flavor and enzymatic browning. As early as 1920 Onslow *(6)* demonstrated, without isolation, the presence of a phenolic compound in peaches and other fruits which gave a green color with ferric salts. This reaction was considered specific for the ortho-dihydroxy grouping. Lavollay, Sevestre, and Dussey *(5)* reported the presence of catechin in peaches. Catechin, in a preparation contaminated with pigments, was identified by oxidation to the cyanidin pigment.

One of the main difficulties encountered in the identification of natural phenolic compounds in fruit tissues has been the lack of satisfactory methods of isolation. One of the principal factors which prompted this investigation was the desire to develop satisfactory methods of isolation. When isolated in reasonably pure form, the rates of enzymatic oxidation of these natural substrates can also be more readily investigated.

This paper describes the use of a synthetic anion exchange resin for the isolation of tannins from peaches. Ion exchange resins have been successfully employed in the separation and purification of many biological materials. The advances made in the field of ion exchange were recently reviewed by Kunin *(3)*.

In preliminary experiments, it was found that phenolic substances such as catechol, tannic acid, and chlorogenic acid were readily adsorbed by anion exchange resin. These substances differ in acidity as well as complexity of the molecule but all were readily adsorbed. The results of these preliminary experiments indicated that an anion exchange resin might possibly be applied in the isolation of phenolic compounds from peaches. Many variations were tried in the use of the resin for isolation of the tannins but only the method which gave the best results is presented in this paper.

EXPERIMENTAL PROCEDURE

Elberta and J. H. Hale peach varieties were used in this investigation. The extraction was carried out directly on the fresh fruit in the case of the Hale peaches. The Elberta peaches were peeled, halved, and dipped in 1500 p.p.m. sulphur dioxide as sodium bisulfite to prevent enzymatic oxidation of the tannins. The halves were then packed in No. 10 size tin cans, sealed, frozen, and stored at −20.6°C.(−5°F.). The tannins were then extracted at a later date.

Tannin extracts were prepared from the frozen peaches by blending 100 g. portions with 300 ml. of 80% ethanol in a Waring blendor for five minutes. The extracts were

[a] Scientific Journal Series No. 334, Colorado Agricultural Experiment Station, Colorado A & M College, Fort Collins, Colorado.

[b] Presented before the Tenth Annual Meeting of the IFT, Chicago, Illinois, May 24, 1950.

then centrifuged to remove pulp and precipitate. This procedure was more satisfactory than filtration. After decantation, the alcohol and part of the water were removed by distillation under reduced pressure. The resulting water extract was then again centrifuged to remove water insoluble material. A yellowish extract having a pH of 3.8 to 4.0 was obtained. Approximately 100 p.p.m. sulphur dioxide as sodium bisulfite was added to the extract to prevent auto-oxidation of the tannins. The extract was then adjusted to pH 7 by the addition of 5*N* sodium hydroxide solution. The extract was also de-aerated by the application of vacuum. The tannin extract was then passed through the anion exchanger which readily adsorbed the tannins and allowed the organic acids to pass through. Kunin *(4)* showed that adsorption of organic acids such as citric acid was very slight at pH 7 when using a weak basic type anion exchanger. He also stated that the anion exchange abilities of various anions depended upon the structure, size, and valence of the anion, and upon the type of salt formed.

Duolite A-2 resin was used in the first experiment but was later replaced by Duolite A-4. Both were satisfactory. Two sizes of columns were used. The smaller column was 1 inch (2.54 cm.) in diameter and contained 30 g. of dry resin. The volume of wet resin was approximately 75 ml. The larger column was 1.5 inches (3.8 cm.) in diameter and contained 80 g. of dry resin or a wet volume of 225 ml.

The resin was put through several cycles of exhaustion with 5% sulphuric acid and regeneration with 5% ammonium hydroxide before it was used. It was then thoroughly washed with water and finally backwashed until the wash water retained a neutral pH.

Adsorption of Tannins. The tannin extract at pH 7 was percolated through the resin column at a rate of flow to maintain a 10-15 minute contact period with the resin. The rate of adsorption of tannins was followed by testing the effluent with Folin-Denis phenolic reagent as described by Rosenblatt and Peluso *(8)*. At the above rate, the initial adsorption was greater than 90%. As the resin became exhausted with the tannins, the adsorption became progressively less. The amount of extract necessary to exhaust the resin in the smaller column was of the order of 1000 to 1500 ml. containing 1500 to 2000 mg. of tannin as tannic acid. This amount was proportionately larger for the larger column. Approximately 70 to 80% of the tannin was adsorbed under these conditions. The change in pH was also followed during adsorption. At the initial stage, the pH of the effluent was greater than nine. As the adsorption became less, the pH also became less. This change in pH indicates that the adsorption process was an ion exchange process. As the extract passed through the resin it changed from a brown color to a light lemon color.

After adsorption, the resin was washed with water and finally with 80% ethanol. The tannins were then eluted with 80% ethanol containing 2% sulphuric acid. The rate of flow used for the elution was about twice the rate used in the adsorption process. The rate of elution of the tannins was followed by testing the eluate with Folin-Denis phenolic reagent. As soon as significant quantities of tannins appeared in the eluate, the collecting container was changed, a portion of the tannins was recovered at their natural pH, and a second portion recovered was acid with sulphuric acid. For this reason it became necessary to follow the pH of the eluate during the elution. As soon as the eluate became more acid than pH 4.5, the collecting container was changed to collect the acid portion. When significant quantities of tannins appeared in the eluate, a change in color was observed. With low concentration of tannin the solution was yellow and it gradually changed to red or reddish brown as the tannin concentration increased.

The neutral portion of the eluted tannins was concentrated to a light sirup under reduced pressure and then finally dried to a powder at 40°C.(104°F.) in a vacuum oven.

The following method was used to recover the tannins from the fraction which was acid with sulphuric acid: A saturated solution of neutral lead acetate was carefully added until the pH was raised to about 2.0 and then the mixture was centrifuged to remove the lead sulfate precipitate. After decantation, the pH of the tannin solution was adjusted to pH 8 and more lead acetate was added to completely precipitate the tannins. The mixture was centrifuged and after decantation the precipitate was washed three times with 70% alcohol. A small amount of 70% alcohol was then added to make a slurry with the precipitate. Then 1*N* sulpuhric acid was slowly added to decompose the lead tannin complex. The pH of the mixture should not decrease to below 3.5. The lead sulfate thus formed was removed by centrifugation. The decantate containing the

tannins has a slight excess of sulfuric acid. This excess sulphuric acid was removed by adding small amounts of anion exchanger which previously had been exhausted with peach tannins by treating it with peach extract. This treatment was carried out merely by adding the exchanger and stirring until the pH of the mixture reached about 4.5. The resin was then removed by filtration and the tannin was recovered from the alcoholic solution in the same manner as described for the tannins which were eluted in neutral solution. It is important to remove all of the free sulphuric acid otherwise polymerization of some of the tannins may take place during the drying process.

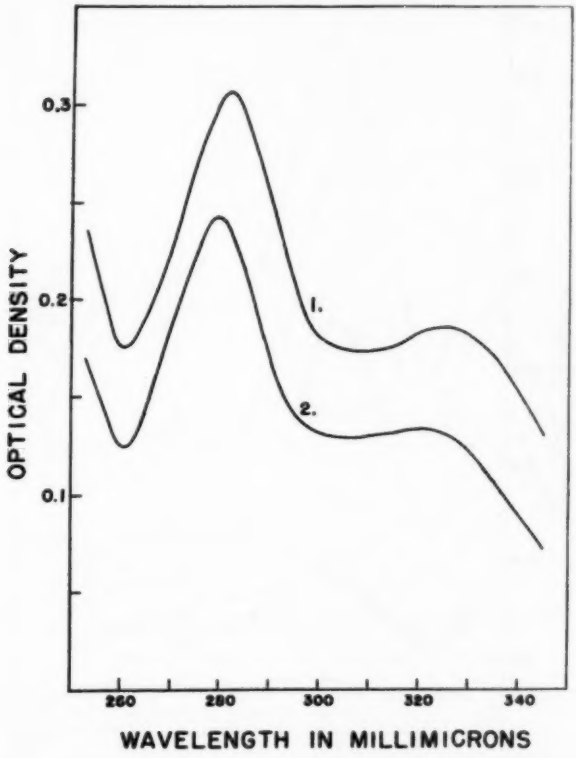

FIG. 1. Comparison of the ultraviolet spectra of lead-precipitated Elberta peach tannins (1) and isolated Elberta peach tannins (2).

The crude tannin preparations obtained were ordinarily reddish brown in color. The J. H. Hale tannin preparations were somewhat darker in color due to greater concentration of anthocyanin pigment. The J. H. Hale peaches contain more anthocyanin pigment than Elberta peaches.

The tannin preparations were subjected to characterization and identification by application of paper chromatography and ultra-violet absorption techniques. The paper chromatography was carried out on Whatman No. 1 filter paper using three different developing solvents, namely, 77% ethanol, butanol-acetic acid-water, and pyridine-

butanol-sodium chloride. The ultraviolet absorption determinations were made on a Beckman Model DU Quartz Spectrophotometer. The Evelyn Colorimeter was used for the measurement of optical densities with the Folin-Denis reagent.

RESULTS AND DISCUSSION

The yields of crude peach tannins obtained were somewhat erratic. The relative amounts of tannins in the neutral and acid effluents were

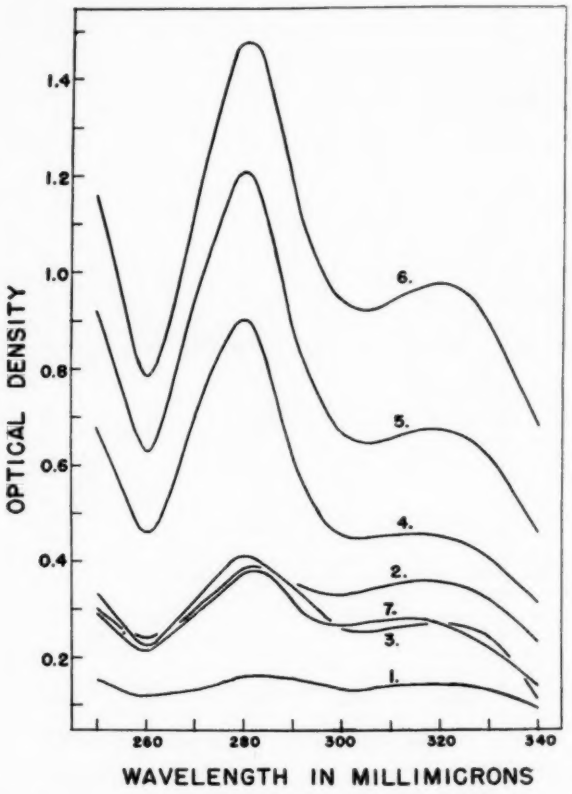

FIG. 2. Change in the ultraviolet spectra of eluate during elution. 0.1 ml. eluate diluted to 4 ml. No. 6 and 7 are acid with sulfuric.

also variable. The 2% sulphuric acid in 80% ethanol did not completely elute the adsorbed tannins. The variables which may influence the yields are not well understood. In general, the capacity of the exchanger decreased with use. Apparently, part of the tannins react with the resin in such a manner that they are not eluted by the acid. The fact that the

resin grows progressively darker in color is evidence for this reaction. Although the capacity of the resin decreases with use, the percent of tannin adsorbed which can be eluted actually increases. Approximately 50 to 60% of the total tannin adsorbed was readily eluted. When sodium hydroxide was used in place of ammonium hydroxide as a regenerant, smaller yields were obtained.

Johnson, Foreman, and Mayer *(2)* described a method for the determination of ultraviolet (UV) absorption of phenolic substances from various fruits. The similarity of UV absorption curves of Elberta phenolic

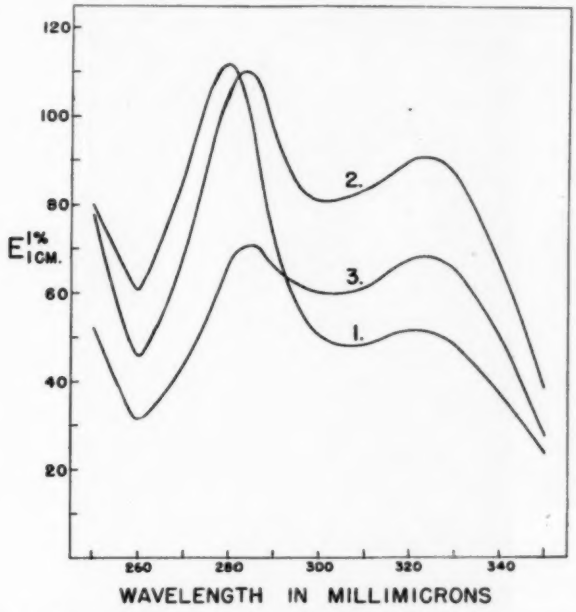

FIG. 3. Ultraviolet spectra of Elberta peach tannin isolates. 1, Eluted neutral; 2, eluted acid; and 3, another peach tannin isolate.

substances as determined by this method and of the isolated peach tannins are shown in Figure 1. A major absorption band is produced at 280 mμ and a minor band at 324-326 mμ. Two different types of substances are responsible for the two bands. Figure 2 shows the UV absorption of the eluate sampled every 15 minutes during the elution. It will be noted that the relative amount of substance producing the 326 mμ band was more concentrated in the acid portion of the eluate. Sample No. 6 was acid with sulphuric. A similar situation is also shown in Figure 4, where the $E^{1\%}_{1cm.}$ values for three Elberta peach tannin preparations are plotted. Curve 2, for the tannins eluted acid, contains more of the substance having the

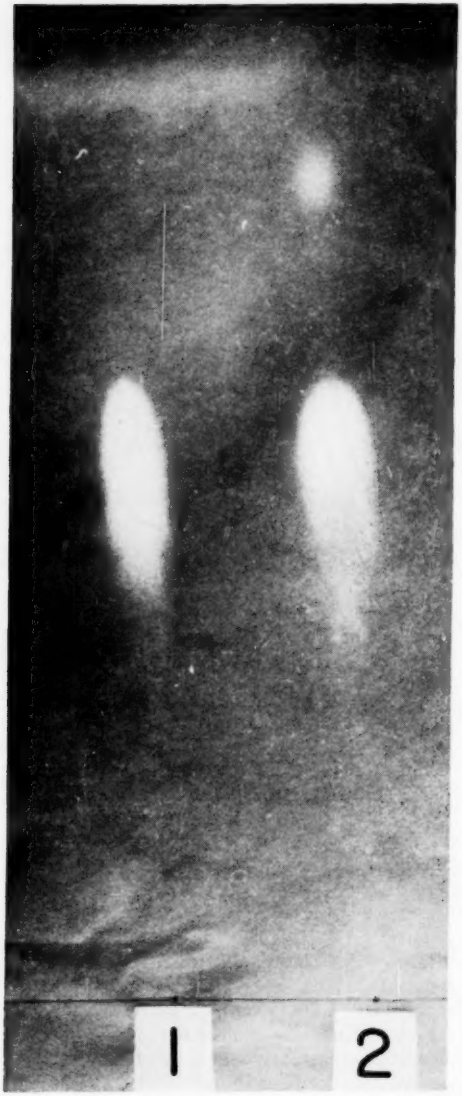

FIG. 4. Fluorescent spots of chlorogenic acid (1) and peach caffetannin (2).

326 mμ absorption band than the tannins eluted neutral. The tannin preparation represented by Curve 3 was found to contain a small amount of free sugars by use of paper chromatography. Butanol-acetic acid-water solution was used as the solvent and the sugars were identified by spraying with aniline hydrogen phthalate reagent, which was described by Partridge *(7)*. The fraction eluted neutral (Figure 3, Curve 1) had only traces of free sugar and the fraction eluted acid had no free sugar.

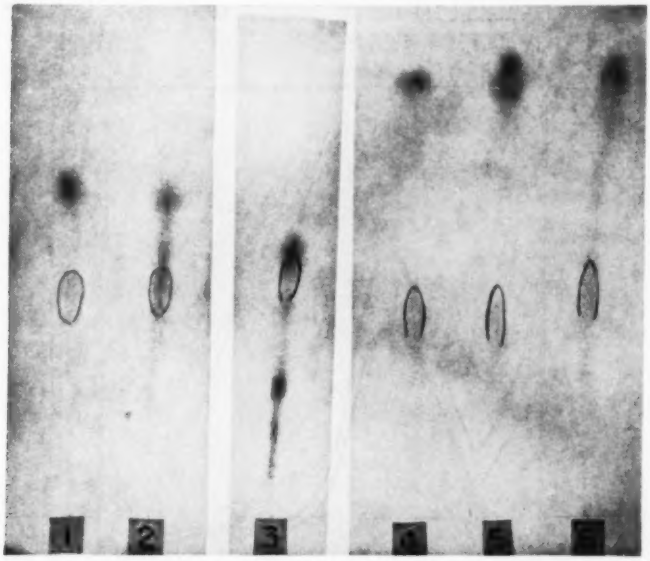

FIG. 5. Papergrams sprayed with 3,4-dinitrosalicylic acid reagent. 1. Chlorogenic acid and D-catechin, and 2. Elberta tannins resolved with 77% ethanol. 3. Elberta tannin resolved with butanol-acetic acid-water. 4. Chlorogenic acid and D-catechin. 5. and 6. Elberta tannins resolved with pyridine-butanol-salt solvent. The outlined spots are the fluorescent chlorogenic acid spots.

Paper chromatography and ultraviolet absorption techniques were used in characterization and identification of tannins in the isolated mixtures. The ascending method of paper chromatography was used with three different solvents. The irrigating solvents used were 77% ethanol, butanol-acetic acid-water, and pyridine-butanol-sodium chloride which was described by Evans, Parr, and Evans *(1)*. Butanol-acetic acid-water solvent was used by Wendler and Gage *(10)* for the separation of flavonoid pigments. After development with the various solvents and drying of the papergram, the location of the tannin spot was obtained by two methods. The papergrams were observed under ultraviolet light having maximum illumination at 365 mμ before and after treating the paper with ammonia fumes. This was followed by spraying the papergram with a saturated solution of 3,5-

dinitrosalicylic acid (henceforth referred to as DNSA) in 50% alcohol containing 2% sodium hydroxide. The tannins give a color reaction with this reagent without heating the papergram. (See Appendix.)

When the papergram was exposed to UV light, one of the substances emitted a vivid blue and greenish yellow fluorescence upon treatment with ammonia fumes. Chlorogenic acid behaves in exactly the same manner and has the same R_f values as this substance with all three solvents. With both substances DNSA gave a bright yellow color which gradually disappeared. The fluorescent spots of chlorogenic acid and the similar substance from peach tannin is shown in Figure 4.

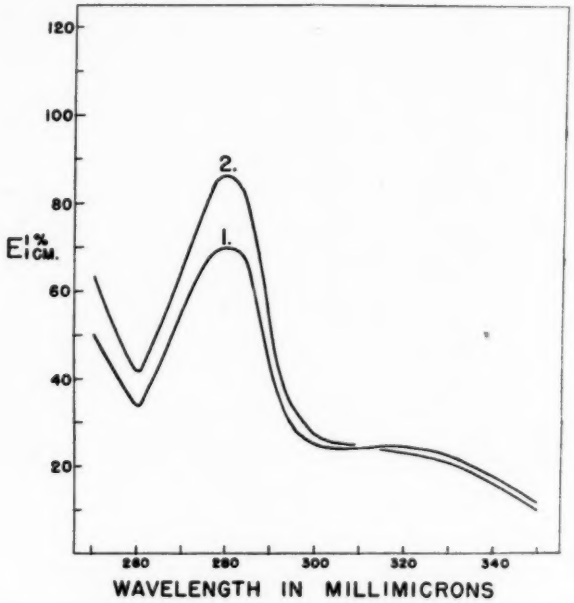

FIG. 6. Ultraviolet spectra of J. H. Hale tannin isolates. 1, Eluted neutral; and 2, eluted acid.

Another substance became visible under UV light when the papergram was treated with ammonia vapor. This substance fluoresced blue but fluorescence was rather weak. It has characteristics very similar to those of D-catechin and has the same R_f a D-catechin in all the three solvents used. This spot was more easily identified by spraying with the DNSA reagent. This reagent produced an orange brown color with both substances.

A third substance appeared upon spraying with the above reagent giving a reddish brown color. This substance was somewhat diffused on the papergrams except when pyridine-butanol-sodium chloride was used

as the solvent. It is located between catechin and chlorogenic acid when alcohol is used in the solvent. Using butanol-acetic acid-water solvent, movement is greatly restricted and it forms a diffused streak on lower part of the papergram. With pyridine-butanol-salt, it is located just above catechin. Actually the two spots will touch. The separation of the above substances on three papergrams sprayed with DNSA is illustrated in Figure 5. From left to right, the papergrams were developed with ethanol, butanol-acetic acid-water, and pyridine-butanol-sodium-chloride solvents.

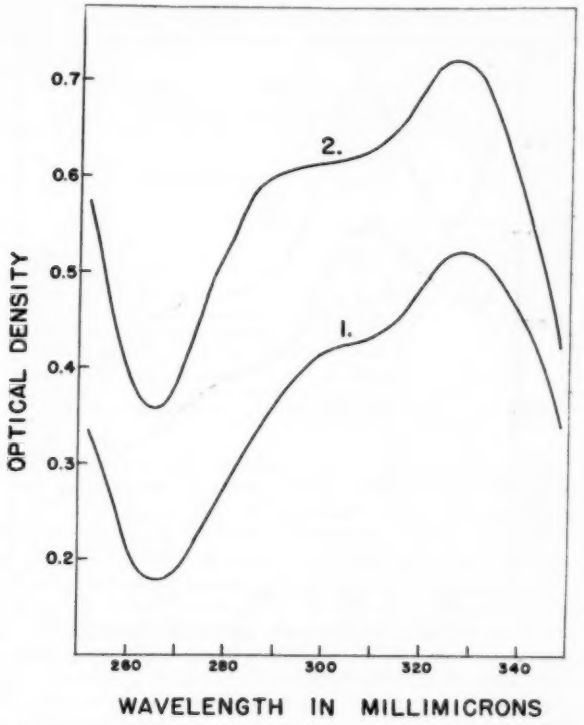

FIG. 7. Ultraviolet spectra of chlorogenic acid (1) and peach caffetannin (2) extracted from papergram.

Numbers 1 and 4 in Figure 5 show the separation of chlorogenic acid and catechin. Numbers 2, 3, 5, and 6 show separation of peach tannins. The spots whose boundaries have been outlined are the ones which fluoresce under UV light, namely, chlorogenic acid and the similar substance present in the peach tannins. Number 6 is a papergram of an Elberta tannin preparation which had very little of the substance which resembled D-catechin. It is also represented by the UV Curve 4 in Figure 2.

By means of paper chromatography, the isolated peach tannins were readily resolved into four components. One component is a caffetannin which resembles chlorogenic acid. Another component greatly resembles D-catechin in its behavior. The third component has properties which resemble phlobatannins (polymers of catechin) and is the main component of the isolated tannins. It contributes the reddish color to the tannin preparations. All three tannin substances give a green color with ferric chloride.

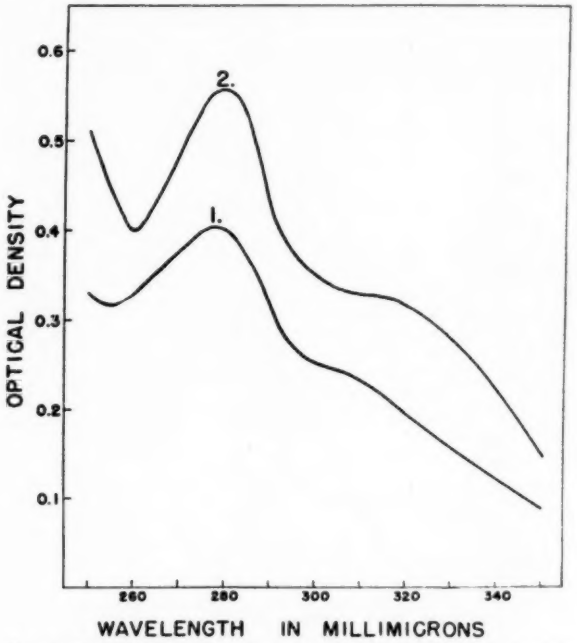

FIG. 8. Ultraviolet spectra of peach tannins extracted from papergram. (1) D-catechin, and (2) phlobatannins.

All the isolated tannin preparations when dissolved in dilute mineral acid, polymerized readily upon heating and formed a precipitate. This behavior is typical of phlobatannins and is referred to as phlobaphene formation.

A fourth component is an anthocyanin pigment which was readily visible when butanol-acetic acid-water solvent was used. J. H. Hale peach tannin preparations contained more anthocyanin pigment than those prepared with Elberta peaches. The R_f value of the anthocyanin pigment in butanol-acetic acid-water solvent and its high partition coefficient between amyl alcohol and 1% aqueous HCl indicate that the pigment is a

3 monoglucoside of cyanidin. The pigment appeared to be completely transferred into the alcoholic phase upon the addition of sodium chloride. UV absorption spectra of the isolated J. H. Hale peach tannins are shown in Figure 6. The fraction eluted neutral was found to contain small amounts of free sugars. However, it could be shown that this fraction contains no free sugar when the resin column is thoroughly washed with 80% ethanol before eluting with acidified 80% ethanol. Free sugars were detected by paper chromatography which showed the presence of sucrose, glucose, and traces of fructose. The tannin eluted acid had no free sugars. The absorption spectra and paper chromatogram revealed that the J. H. Hale tannin preparations contained relatively less caffetannin than the Elberta tannins.

TABLE 1

Characterization of Various Samples

Sample No.	Description of sample	$E^{1\%}_{1cm.}$ at 280 mμ	Optical density with Folin-Denis reagent, (0.1 mg./100 ml.)
1	D-Catechin	132	0.180
2	Tannic acid	.....	0.168
3	Chlorogenic acid	.....	0.130
4	Elberta tannins, eluted neutral (Figure 3, Curve 1)	112	0.126
5	Fraction of above (4) soluble in acetone	120	0.132
6	Fraction of above (4) insoluble in acetone	98	0.101
7	Elberta tannins eluted acid (Figure 3, Curve 2)	106	0.097
8	Elberta tannins (small amount of free sugars)	70	0.060
9	J. H. Hale tannins eluted neutral (Figure 7, Curve 1)	70	0.070
10	J. H. Hale tannins eluted acid (Figure 7, Curve 2)	86	0.078

Sufficient quantities of the three main components of the isolated tannin (not anthocyanin pigment) were separated on papergrams and extracted from the paper with 70% ethanol so that UV absorption spectra could be determined on these substances. Figure 7 shows the similarity of UV spectra for chlorogenic acid and the peach caffetannin. To obtain good separation of chlorogenic acid from the other peach phenolic substances, it was found necessary to use two-dimensional paper chromatography. Butanol-acetic acid-water (40-10-50) and 76-78% ethanol were the two developing solvents used. UV light with maximum transmission at 365 mμ was used to locate the chlorogenic acid spot on the papergram. Maximum absorption at 324-326 mμ was obtained for both substances. Russell *(9)* stated that caffetannins are closely related to or even identical with chlorogenic acid. The UV spectra of the other two substances are given in Figure 8. Both these substances have an absorption band at 278-280 mμ.

Purity of Isolated Tannins

The peach tannins were obtained in relatively pure form by this method of isolation. Some of the tannin preparation eluted neutral contained small amounts of free sugars. The tannin fractions recovered from the acid portion contained no free sugars. However, upon hydrolysis of this fraction with 6*N* HCl, tests for glucose were obtained except in the case of

J. H. Hale acid fraction. The relatively high degree of purity of the tannin preparations is shown by the UV extinction coefficients and the blue color formation with Folin-Denis reagent. These results are given in Table 1.

The fraction soluble in acetone (Sample No. 5, Table 1) had the highest extinction coefficient and it approached that of D-catechin. It also gave the highest color with Folin-Denis reagent. This fraction is slightly pinkish in color and is richer in the tannin substance which resembles D-catechin. This fraction apparently is not associated with a carbohydrate. The reddish colored fraction of the isolated tannins is only slightly soluble in acetone and is associated with a carbohydrate since glucose appears upon hydrolysis with hydrochloric acid.

The caffetannin makes up only a relatively small fraction of the total tannin. This is borne out by the UV absorption spectra since chlorogenic acid has a much higher extinction coefficient (530) at its maximum absorption at 324 mμ than the tannins which have their maximum absorption at 280 mμ.

SUMMARY

A method employing synthetic anion exchange resin for the isolation of peach tannins is described. Paper chromatography and ultraviolet absorption techniques were used in the characterization and identification of the phenolic substances in the isolated material.

The isolated tannins contained 1, D-catechin; 2, a reddish colored substance probably polymeric derivatives of catechin, namely, phlobatannins; 3, a caffetannin which was identified as chlorogenic acid; and 4, an anthocyanin pigment, apparently the 3-monoglucoside of cyanidin.

Appendix Added February 19, 1951

Since the presentation of this paper methods of detecting the phenolic compounds on the papergram have been improved. Two-dimensional chromatography using butanol-acetic acid-water (40-10-50) and 76-78% ethanol as developing solvents improved separation. Diazotized sulfanilic acid (Pauly reagent) was found to be more sensitive for detecting phenolic compounds than 3,5-dinitrosalicylic acid or ferric chloride reagent. The Pauly reagent gives a pinkish color with chlorogenic acid and yellowish tan with the other phenolic compounds of peaches. The use of this reagent for detecting phenolic compounds was described by Evans, et al. *(1)*.

Folin-Denis phenolic reagent diluted with an equal volume of 95% ethanol was also found to be very useful for detecting phenolic compounds.

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HEAT PENETRATION DURING BAKING AND QUALITY OF SHORTENED CAKES VARYING IN pH VALUE[a]

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Cakes in which part or all of the liquid consists of acid fruit juice have been observed to have fine grain and even texture, while those containing excess soda have coarse grain and open texture. The characteristic grain and texture of the latter have been attributed in part to the increased dispersion of the gluten by the unneutralized soda *(11)*.

The quantity of gluten in cake flour is so small and the effect of the relatively large amounts of fat and of sugar on any gluten present in the flour are so great that it seems likely that there are other factors which might help to account for the effects of either excess soda or acid on cake quality.

Rapid heat penetration makes for improved texture of cakes made with fat, whether that be due to higher baking temperatures *(14)*, or to greater efficiency of baking utensils *(5)*. It was the purpose of the work here reported to study the rate of heat penetration during the baking of shortened cake batters varying in pH value and to correlate this with certain quality characteristics of the finished cakes.

EXPERIMENTAL PROCEDURE

Formula and Method of Combining Ingredients. The basic formula for the plain cake batter and the method of mixing were the same as those used in a previous study conducted on cakes with fat *(5)*. To effect variations in the pH of the cake batter, two levels of baking soda and two of cream of tartar were added to the plain cake formula. In each series or replication there were five cakes: one containing the 4.5 grams of tartrate baking powder called for in the basic formula, and four cakes containing, in addition to the baking powder, either 1 gram of baking soda, 0.5 gram of baking soda, 0.5 gram of cream of tartar, or 1 gram of cream of tartar.

pH of Batter. The hydrogen ion concentration of the cake batter was measured with the glass electrode of a Beckman pH meter, Model G. Readings were made on the undiluted, unstirred batter immediately after the batter was mixed. The pH value for any one sample was the average of two readings which checked to within 0.1 pH reading or less, as suggested by McKim and Moss *(12)*.

Specific Gravity of Batter. The specific gravity of the batter was determined by the method outlined by Dunn and White *(7)*.

Baking. Two hundred twenty-five grams of batter were panned into rectangular shaped, tinned iron pans with a capacity of 650 milliliters. Baking was done in an electric oven thermostatically controlled at 185°C. ± 1°, and heat penetration readings were taken as previously described. The cakes were baked two minutes longer than the time required to reach maximum internal temperature.

After removal from the oven the cakes were allowed to cool in the pans to 60°C. (140°F.), after which they were taken from the pans and cooled to room temperature. They were then wrapped in waxed paper and stored in a tight container until the tests were made on the following day.

Cake Volume. Using a miter box, a slice was cut from each end of each cake and a third cut was made crosswise through the center. Tracings were made around each end slice and around the facing edges of the cut through the center of the cake. The

[a] Supported in part by a grant-in-aid from General Research, Oregon State College.

standing index to the volume of each of the four tracings was measured by a polarizing planimeter *(13)*.

Scoring the Cakes. A panel of three judges rated cut slices from each of the five cakes for crumb quality approximately 24 hours after they were baked. The cakes were ranked in descending order for whiteness, fineness of cells, uniformity of cells, sweetness, flavor other than sweet, moistness, tenderness, and over-all desirability.

Statistical Analysis. Statistical analysis of the data on the specific gravity of the batter, the length of the baking period, and the subjective scores was done under the direction of Dr. Jerome C. R. Li of the Department of Mathematics of Oregon State College.

RESULTS AND DISCUSSION

Given in Table 1 are the average pH values of the five cake batters used. The specific gravity of the batter varied from 0.802 for that having 1 gram of added soda to 0.843 for that with 1 gram of added cream of tartar. Statistical analysis of the data showed a significant difference in specific gravity ($F = 36.23$ with four and 20 degrees of freedom) due to treatment. At the 5% level a difference of 0.009 in specific gravity is significant. The batters containing added soda were significantly lighter than either those containing additions of cream of tartar or those containing baking powder alone, the low specific gravity of the batters containing added soda doubtless being due to the evolution of a larger amount of carbon dioxide. The specific gravity of the batter to which one gram of soda was added was significantly lower than that of the batter to which 0.5 gram of soda was added.

TABLE 1

Means of Certain Characteristics of Cakes Varying in pH Value [b]

Added Ingredient	pH of Batter	Specific Gravity of Batter [c]	Baking Time	Standing Index to Volume [d]
			min.	*sq. in.*
1 gram cream of tartar	5.31	0.843	28.5	17.96
0.5 gram cream of tartar	5.47	0.839	28.8	17.98
None	5.53	0.841	28.8	18.05
0.5 gram baking soda	6.08	0.815	28.6	19.05
1 gram baking soda	6.34	0.802	29.5	19.56

[b] Based on six replications.
[c] A difference in specific gravity of 0.009 is significant at the 5% level.
[d] A difference in treatment mean of 0.27 is significant at the 5% level.

Baking Time. The lengths of baking periods for cakes made from the basic formula alone or with the additions of either 0.5 gram of cream of tartar, 1 gram of cream of tartar, or 0.5 gram of soda did not differ. The baking time for cakes made from batter containing 1 gram of soda averaged approximately one minute longer than that for the other four batters and an analysis of variance of the data for the baking time for the batters containing 1 gram of added soda against the four other treatments indicated that this difference was significant ($F = 6.45$ with one and 20 degrees of freedom). From a practical standpoint, of course, a difference of one minute in baking time is not important, although from the standpoint of the effect on cake quality it may be.

Heat Penetration Curves. The heat penetration curves for the batter made from the plain formula and for batter containing either 0.5 gram or 1 gram of cream of tartar are similar, although the temperature in

cakes with added cream of tartar averaged two or three degrees higher than in the plain cake formula during most of the baking period. After the first minute or two in the oven, there was a rapid and regular rise in temperature in these cakes for the next twenty minutes, giving a heat penetration curve that is practically a straight line, except for a slight flattening between the sixteenth and twentieth minute interval of baking.

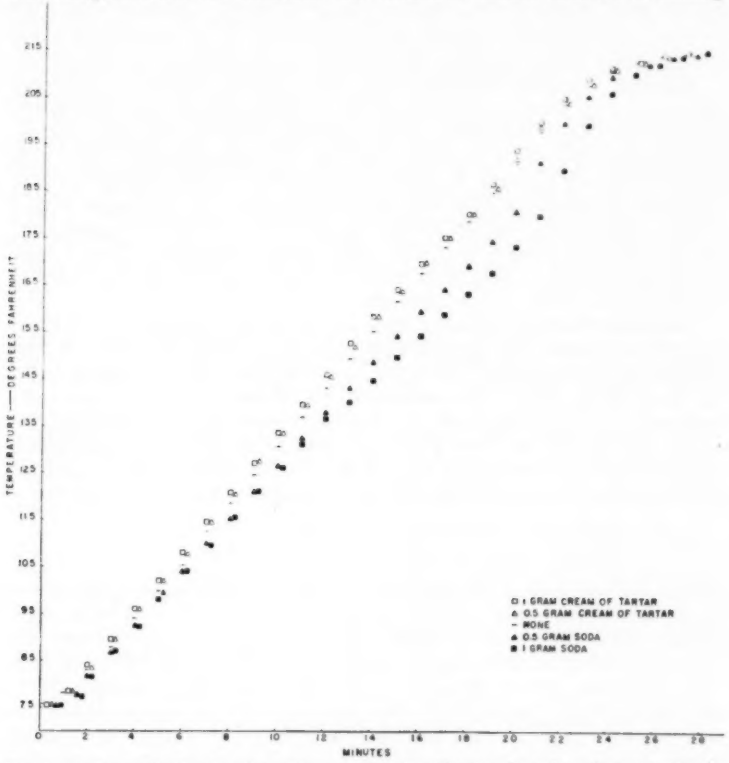

FIG. 1. Heat penetration in cake batters varying in pH value (based on six replications).

This slight flattening of the heat penetration curves [between 60° and 85°C.(140° to 185°F.)] is probably the result of increased heat absorption due to rapid gelatinization of the starch in the batter *(1)*.

In the heat penetration curves for batters with added soda, the flattening was much more pronounced than in those battters with lower pH values. The heat penetration curve for the batter containing 1 gram of soda showed a decided flattening beginning at the end of the first twelve minutes in the oven. The slower transfer of heat into these batters is doubtless due chiefly to the large amount of carbon dioxide evolved, as indicated by the lightness of the uncooked batters.

At the end of twenty minutes in the oven the temperature of the batter containing 0.5 gram of soda began to rise abruptly and continued to do so for three or four minutes, after which it began to level off. In the case of the batter containing 1 gram of soda, the rise was somewhat less abrupt and came approximately one minute later in the baking period. In both instances the sharp rise in the heat penetration curves occurs at approximately 85°C.(185°F.). The cooling effect of water rapidly evaporating from the crust possibly accounts for the leveling off of all the heat penetration curves during the last few minutes of the baking period *(1)*.

Volumes. Statistical analysis of the data (Table 1) showed a significant difference in the volumes of the cakes ($F = 67.14$ with four and 20 degrees of freedom) due to treatment. Cakes containing added soda were significantly larger than cakes from the other three treatments, those made with one gram of soda yielding significantly larger cakes than those made with 0.5 gram. It will be recalled that the specific gravity of the batter with 1 gram of added soda was the lowest, followed by that with 0.5 gram, and this in turn by batters from the other three treatments. Thus a high correlation between lightness of batter and lightness of cake, as reported by Dunn and White *(7)*, is apparent.

Subjective Scores. The cakes were scored the day after baking by three experienced judges. The judges were asked to rank the five cakes in each replication in descending order for (1) whiteness, (2) fineness of cells, (3) uniformity of cells, (4) sweetness, (5) flavor, other than sweet, (6) moistness, (7) tenderness, and (8) over-all desirability. The cake that was at the head of the list for a particular item on the score card got a rank of 5, that at the bottom of the list a rank of 1. The ranks were transformed into normal scores using Table XX of Fisher and Yates *(9)* as follows:

Rank	5	4	3	2	1
Normal Score	1.16	0.50	0.00	−0.50	−1.16

Analysis of variance of the data showed that the addition of cream of tartar or soda to the batter caused a significant difference in each of the eight characteristics for which the finished cakes were ranked by the judges. The analysis yielded the following F values with four and 60 degrees of freedom: whiteness, 113.76; fineness of cells, 72.08; uniformity of cells, 26.66; sweetness, 101.24; flavor other than sweet, 108.46; moistness, 41.41; tenderness, 9.80; and over-all desirability, 86.11. Cakes made with added cream of tartar were rated above those from the basic formula and these in turn were ranked above cakes made with added soda.

The more acid the batter the whiter, more moist, and more tender the cake crumb. With increasing acidity, the cakes were also rated sweeter and better in flavor other than sweetness. As shown by Fabian and Blum *(8)*, the effects of sub-threshold concentrations of acid on sucrose may account for the increasing sweetness of the cakes with increasing hydrogen ion concentration. Aside from the question of the objectionable taste of soda in the cakes to which it had been added, raising the pH of the cakes resulted in an unpleasant, "eggy" odor. No doubt the preference for the flavor of cakes with lower pH values was due in part to the greater retention of vanilla flavor during the baking period *(10)*.

Cakes made from batter containing the top level of cream of tartar had finer and more uniform grain than did cakes made from the basic formula alone, while those containing added soda had a decidedly coarse and uneven grain. The lengths of the baking times and the heat penetration curves for the cakes may throw some light on the effects of the various treatments on texture of the cakes. The batter containing 1 gram of added soda required a significantly longer time to bake than did that from any of the other four treatments. This could account for the low quality of the crumb of these cakes, as slow heat penetration has been shown to produce cakes of inferior crumb quality *(5, 14)*.

Cakes from batter containing 0.5 gram of soda took no longer to bake than did cakes from the basic formula alone or with either of the two levels of added cream of tartar; however, they did require two minutes longer to reach the temperature at which there was a rather sharp upward break in the heat penetration curve. Cakes from batter containing 1 gram of soda required three minutes longer to reach this point [85°C.(185°F.)]. This is the approximate temperature which, according to Caesar *(3)*, marks

TABLE 2

Average Normal Scores for Cakes Varying in pH Value *

Added Ingredient	Whiteness	Fineness of Cells	Uniformity of Cells	Sweetness	Flavor, non-sweet	Moistness	Tenderness	Over-all Desirability
1 gram cream of tartar	0.83	0.76	0.73	0.85	0.88	0.88	0.57	0.68
0.5 gram cream of tartar	0.53	0.45	0.36	0.52	0.58	0.40	0.36	0.62
None	0.31	0.45	0.27	0.27	0.15	0.16	0.00	0.33
0.5 gram baking soda	−0.50	−0.54	−0.52	−0.47	−0.46	−0.43	−0.25	−0.47
1 gram baking soda	−1.16	−1.13	−0.85	−1.16	−1.16	−1.01	−0.67	−1.16
Minimum significant difference at the 5% level	0.22	0.27	0.39	0.23	0.22	0.32	0.44	0.24

* Based on six scorings by each of three judges.

the peak in the gelatinization of a 20% wheat starch paste, roughly the percentage of starch in the cake batters under consideration. Before the starch in the cake batter gels and while still fluid, gas cells are carried about with convection currents and coalesce readily, with a tendency for some of the larger gas cells to absorb the smaller ones *(4)*. Delay in the gelatinization of the starch in the cake batters with added soda and the resultant extra two or three minutes of agitation of the warmed and still mobile batter, together with an opportunity for increased expansion of the batter cells, apparently are critical as far as texture is concerned. These heat penetration curves lend support to the theory that the tendency to coarseness and lack of uniformity of the cell structure of cakes made with added soda may be due in part to the slower rate of heat penetration during baking and to the delay in the gelatinization of the starch.

Delay in the coagulation of the egg protein may be another factor responsible for the poor texture of cakes with added soda as compared

with those containing added cream of tartar. Acid has been shown to lower the initial coagulation temperature of egg protein *(2)*, and to increase its rate of coagulation, while alkali has the opposite effect as noted by Chick and Martin *(6)*. Thus the addition of cream of tartar or soda may influence the texture of cake by affecting the temperature at which the batter begins to set as well as the length of time required for the final stages.

SUMMARY

The rate of heat penetration during the baking of shortened cake batters of different hydrogen ion concentrations has been studied. Variations in pH of the batter were effected by the additions of either soda or cream of tartar to a basic cake formula.

The length of the baking period was the same for all cakes except for those containing the higher amount of soda, which averaged approximately one minute longer. Heat penetration curves for cakes made from the basic formula alone and with added cream of tartar were similar, the internal temperature in the cakes with 1 gram of added cream of tartar running slightly higher than in those with 0.5 gram and the internal temperature of the latter in turn running slightly higher than in the cakes from the basic formula. Heat penetration in cakes with added soda was decidedly slower, doubtless due to the larger amount of carbon dioxide evolved in these batters.

The possible relationship between the rate of heat penetration during the baking period and certain quality characteristics observed in the finished cakes has been discussed.

Acknowledgments

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THE PRESENCE OF ENTEROCOCCI IN SPRAY-DRIED WHOLE EGG POWDER[a]

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In studies on the microbial flora of high moisture (4 to 6%) spray-dried whole egg powder, the frequent appearance of pin-point colonies on the different agar-plate media was noted. On microscopic examination, these were shown to be gram-positive cocci, singly, in pairs, and in short chains. It was also noted that cocci and paired cocci predominated in those samples of powder having a high direct microscopic count.

This report summarizes the results of a detailed study of 74 isolations made from the pin-point colonies. Growth rate and growth acceleration studies were made in liquid egg in an effort to interpret the possible behavior of these organisms during the egg drying operation.

EXPERIMENTAL PROCEDURE

Isolations were made from glucose-tryptone extract agar, wort agar, and Bacto-Dextrose Tryptone agar serial dilution plates used in the microbial examination of spray-dried whole egg powder *(4)*. After purification, all cultures were maintained on 5% horse-blood agar slants.

The temperature limits of growth were determined in litmus milk. The cultures were adjusted to incubation temperature immediately after inoculation, and then incubated for one week at 10° and 45°C. (the latter in a thermostatically controlled water bath). Simultaneously, controls for each culture were inoculated at 37°C. Growth at the test temperature was noted from daily observations of characteristic reactions of the culture medium. In addition, as an additional check, all tubes were subcultured to horse-blood agar at several intervals during the test period.

Thermal resistance was determined by inoculating 5 ml. of sterile skimmed milk with 0.5 ml. of the test culture, and heating for 30 minutes at 60°C. in a thermostatically controlled water bath. The tubes were then incubated at 37°C. and survival noted by subculture to horse-blood agar.

The ability to grow in media containing 6.5% NaCl was tested in 0.5% dextrose broth to which sufficient NaCl was added aseptically to make a final concentration of 6.5%. Tubes of the media were inoculated with the test cultures, incubated at 37°C. for at least one week, with periodic observations to record and observe growth.

Growth in media of high pH was determined in 0.5% dextrose broth adjusted aseptically to pH 9.6 with NaOH immediately before inoculation. The pH was checked daily during the test period in order to note maintenance of test pH. Cultures were grown in the medium for at least one week, with periodic observations to record and observe growth.

Final pH was determined in 1% dextrose broth. The tubes were incubated for one week at 37°C. Determinations were made on a Beckman pH meter with a glass electrode.

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Sugar fermentations were carried out in meat-extract broth containing 0.5% of the test sugars with brom-cresol purple as the indicator.

The ability to liquefy gelatin was determined in gelatin broth incubated at least 3 weeks at 37°C. Periodically the tubes were refrigerated in order to determine true liquefaction.

Serological grouping was performed on a representative number of strains. Preparation of antigen extract, and the microprecipitin test were carried out according to a modification of the Lancefield technic *(2, 3, 6)*. Strains were tested against streptococcal groups A, B, C, D, E, F, and G sera.

Growth studies of *Streptococci* in liquid egg were carried out in the following manner. Fresh eggs, 24-72 hrs. old, were aseptically broken out. The egg liquid was churned briefly with a mechanical stirrer and inoculated with a 24-hour nutrient broth culture of the test organism incubated at 37°C., or, where indicated, with a composite of several test organisms grown under the same conditions. The inoculated egg was again churned for approximately one minute, and then dispensed in 100-ml. quantities into sterile glass containers which were incubated at the different test temperatures. At stated intervals, viable cell counts were made on glucose-tryptone agar. In order to test acceleration of growth of *Streptococci* in liquid egg, similar experiments were carried out, but the organism used for inoculum was incubated in liquid egg at 45°C. for 15 hours instead of nutrient broth at 37°C. for 24 hours.

The results obtained are shown in Tables 1-3.

TABLE 1

Multiplication of Streptococci (Culture No. 43) in Batches of Liquid Whole Egg Held at Temperatures Ranging from 0° to 55°C.

Holding time, hours	Holding Temperatures						
	0°C. (32°F.)	10°C. (50°F.)	23°C. (73.4°F.)	30°C. (86°F.)	37°C. (98.6°F.)	45°C. (113°F.)	55°C. (131°F.)
	Uninoculated Control						
	<100	<100	<100	<100	<100	<100	<100
	Inoculated						
0	2.2×10^4	2.2×10^4	2.2×10^4	2.2×10^4	2.2×10^4	2.2×10^4	2.2×10^4
1	.....	.....	1.9×10^4	1.6×10^4	1.1×10^4	1.2×10^4	9.1×10^3
2	1.4×10^4	1.7×10^4	1.3×10^4	1.1×10^4	1.3×10^4	1.0×10^4	4.5×10^3
3	.....	.....	1.7×10^4	1.6×10^4	1.2×10^4	2.4×10^4	3.2×10^3
4	1.3×10^4	1.6×10^4	1.4×10^4	1.0×10^4	3.5×10^4	9.2×10^4	2.8×10^3
5	.....	.....	1.6×10^4	2.6×10^4	1.5×10^5	7.4×10^5	2.2×10^3
6	1.1×10^4	1.9×10^4	1.5×10^4	9.7×10^4	8.8×10^5	2.8×10^5	1.3×10^3
24	1.5×10^4	1.7×10^4	1.8×10^5	5.0×10^5	5.8×10^5	1.1×10^5	0
pH after 24 hrs.	7.25	7.28	6.42	6.12	6.00	6.00	6.92

RESULTS

All but two strains grew at 45°C. Seven strains, including the two failing to grow at 45°C., also failed to grow at 10°C. Controls incubated at 37°C. grew satisfactorily. All 74 strains survived heating at 60°C. for 30 minutes.

Of the 74 strains tested, 68 were able to grow in media containing 6.5% NaCl. All 74 strains were able to grow in broth of pH 9.6. The final pH attained in 67 cultures was in the range of 4.0-4.4. The remaining 7 cultures attained a final pH in the range 4.5-5.3.

All 74 strains fermented dextrose and maltose. The majority of strains fermented lactose, sucrose, salicin, mannitol, and trehalose, and failed to

ferment sorbitol, raffinose, and inulin. All but two cultures failed to liquefy gelatin. All cultures produced slight zones of greening around the colonies when examined on horse-blood agar pour plates.

Of 30 strains tested, all gave positive reactions with group D sera, although in several instances the response was weak. None of the strains reacted with any of the other sera tested (A, B, C, E, F, G).[4]

Table 1 illustrates the multiplication of a test strain in liquid egg at incubation temperatures ranging from 0° to 55°C. No increase in viable cells was noted in liquid egg held up to 24 hours at 0° and 10°C. Multiplication was active at temperatures of 23°, 30°, 37°, and 45°C., whereas a decrease in count was observed in liquid egg held at 55°C. Similar results were obtained with other *Streptococcus* strains incubated in liquid egg, as well as with a composite of 10 *Streptococcus* strains (Table 2). In these experiments, the test inocula were made from nutrient broth cultures of *Streptococci* incubated 24 hours at 37°C.

TABLE 2

Multiplication of a Composite of 10 Strains of Streptococci Inoculated Into Churned Fresh Egg and Held for 24 Hours at Various Holding Temperatures

Holding time, hours	Holding Temperatures						
	0°C. (32°F.)	10°C. (50°F.)	23°C. (73.4°F.)	30°C. (86°F.)	37°C. (98.6°F.)	45°C. (113°F.)	55°C. (131°F.)
	Uninoculated Control						
	<100	<100	<100	<100	<100	<100	<100
	Inoculated*						
2	5.1×10^2	5.1×10^2	4.7×10^2	5.2×10^2	4.5×10^2	2.5×10^2	1.2×10^2
4	.....	.....	.....	3.6×10^2	4.5×10^2	1.4×10^4	.....
5	.....	.....	3.5×10^3	8.8×10^3	1.3×10^5	1.0×10^6	.....
6	6.6×10^2	6.3×10^2	4.6×10^3	3.4×10^4	9.5×10^5	5.9×10^6	470
8	.....	.....	3.7×10^4	4.3×10^5	2.0×10^7	8.6×10^7	.....
10	.....	.....	1.7×10^5	5.7×10^6	2.1×10^8	4.4×10^8	.....
12	5.8×10^2	6.6×10^2	7.0×10^5	6.8×10^7	9.1×10^8	9.4×10^8	20
24	5.9×10^2	8.9×10^2	6.6×10^6	6.0×10^8	2.4×10^8	1.9×10^8	0
pH after 24 hrs.	7.72	7.65	6.22	6.15	6.15	6.16	7.18

* Composite used for initial inoculum prepared from nutrient broth cultures of individual strains incubated 24 hours at 37°C. No counts however were made at the 0 hour.

When the test inoculum was incubated in liquid egg 15 hours at 45°C. prior to its addition to the liquid egg, accelerated growth of the strains was noted at the higher temperatures of incubation, 45°, 50°, and even 55°C. up to 6 hours (Table 3). Further incubation at 55°C. through 24 hours resulted in a sharp decrease in viable cell count. Similar results were also obtained under the same experimental conditions with other *Streptococcus* strains and with the composite of 10 *Streptococcus* strains.

[4] We are indebted to Dr. R. Lancefield, Rockefeller Institute for Medical Research, who generously supplied the group sera used in the tests. Dr. Lancefield checked 16 of our strains and confirmed our findings. We also owe thanks to Mrs. A. Gelman, Columbia University School of Public Health, who kindly checked all 30 of our strains and confirmed our results.

TABLE 3

Multiplication of Streptococci (Culture No. 58) in Batches of Liquid Whole Egg Held at Temperatures Ranging from 45° to 55°C.

Holding time, hours	Holding Temperatures		
	45°C. (113°F.)	50°C. (122°F.)	55°C. (131°F.)
0	6.5×10^4	8.7×10^4	8.5×10^4
1	.....	6.6×10^4	1.2×10^5
2	.....	1.0×10^5	2.0×10^5
3	.....	1.3×10^5	2.5×10^5
4	.....	1.1×10^5	3.2×10^5
5	.....	1.8×10^5	4.9×10^5
6	1.3×10^6	2.0×10^5	3.2×10^5
24	7.9×10^6	2.5×10^6	300
pH after 24 hrs	6.22	7.88	7.82

DISCUSSION AND CONCLUSIONS

Morphological, biochemical, and serological findings indicate that the majority of isolates from the pin-point colonies conformed to the pattern for the *Enterococci*; those tested serologically fell into the Lancefield group D, thus confirming their identity. Although variability was observed in some of the biochemical tests, under specified and controlled conditions, the "tolerance" tests recommended by Sherman *(5)* were found to be essentially reliable for identifying the *Enterococci*, especially when grouping sera are not always available for serological confirmation.

Although studies have been made on the microbial flora of spray-dried whole egg powder, attention has been focussed on viable and total bacterial counts, presence of *Salmonella sp.*, *Escherichia coli*, coliforms, and molds. Johns and Berard *(1)* have suggested that the presence or absence of *Streptococci* in egg powder may possibly offer a means of distinguishing between powders of high microbial count caused by faulty plant practices and those caused by poor quality eggs (in which case the predominant organisms are usually rod forms). Their data partially support this view, and it is our impression that it may be true, although it was not possible to analyze our data with this distinction in mind.

As indicated in our experimental data, *Streptococci* multiply in liquid egg. Accelerated growth of the organisms was noted at higher incubation temperatures when the cultures used for inocula were maintained at elevated temperatures. It is possible that this information may be used to predict the results of faulty or lax plant operational activities, as for example the holding of refrigerated tanks containing liquid egg at elevated temperatures and for longer than acceptable periods prior to drying. Such practices might conceivably permit a bacterial build-up of *Streptococci* in a few hours in liquid egg held at improper temperatures, especially where unsatisfactory sanitation and control measures are used in egg-dehydration plants.

In the recognized absence of correlation between the presence of *E. coli*, coliforms, *Salmonella sp.*, and plate counts of viable organisms or direct microscopic counts, consideration might be given to the *Enterococci* as

indices of fecal contamination and/or faulty plant practices. Certainly the validity of the relationship should be explored.

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A STUDY OF CERTAIN FACTORS INFLUENCING THE COMPOSITION OF CALIFORNIA-TYPE SHERRY DURING BAKING

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The characteristic odor and flavor of California-type sherry are developed by heating white wines at temperatures of 48.9° to 60.0°C. (120° to 140°F.) for a period of two to four months. The wine used should contain 20.0 to 21.0% alcohol and 0.2 to 5.0% reducing sugar. The wine is most often stored in an insulated room in redwood or concrete tanks and is heated by coils containing steam or hot water. Occasionally the wine is placed in oak barrels or puncheons in heated rooms. Some California-type sherry is also developed by storing uncooked or partially-cooked wine in oak containers placed outdoors in the sun. More or less aeration accompanies all of these methods. This may result from the air space in the upper part of the containers or from forced aeration by circulation of the wine or even from pumping air through the wine. The value of aeration has not been established. Very little is known about the changes in composition that occur during the baking or about the influence of the original composition of the wine on the rate or extent of the changes resulting from the heating. See, for example, Joslyn and Amerine *(5)*. The experiments reported here were planned to investigate these points.

EXPERIMENTAL

A sherry material (shermat) was prepared from White Riesling, Sauvignon blanc, and Chardonnay dry white wines in the proportions of 2:2:1. The grapes from which the wines were produced were grown at Davis and were quite suited for shermat production. The original total acidity, for example, averaged about 0.55%, as tartaric acid. The three wines were blended, fortified to approximately 20.0% alcohol, close-filtered through a Zeiss pad filter, and divided into nine lots as follows: (1), untreated; (2), calcium carbonate, 3.75 g. per gallon (to reduce the total acidity approximately 0.10%); (3), dextrose, 2%; (4), sulfur dioxide, 100 p.p.m.; (5), citric acid, 6.5 g. per gallon (to increase the total acidity approximately 0.10%); (6), calcium carbonate, 3.75 g. per gallon, and dextrose, 2%; (7), calcium carbonate, 3.75 g. per gallon, and sulfur dioxide, 100 p.p.m.; (8), dextrose, 2%, and sulfur dioxide, 100 p.p.m.; and (9), calcium carbonate, 3.75 g. per gallon, dextrose, 2%, and sulfur dioxide, 100 p.p.m.

The nine lots were then divided into two portions; one half for heating in an unaerated condition and the remainder for heating with weekly aerations. The wine to be heated unaerated was placed in full, tightly-corked one-gallon glass containers. Three pint bottles for each lot were also completely filled and corked. These were used to replenish the gallon-jugs after each sampling. The wine to be aerated during heating was placed in half-filled two-gallon containers. These were aerated once a week by drawing air through them from an ordinary aspirator for two minutes. Three bottles for each of these lots were also half-filled and were used to replenish the wine in the two-gallon containers after each sampling.

The temperature of the room was thermostatically controlled at 49°C. ± 1.1° (120°F. ± 2°). Analyses were made in duplicate on the original wines and on samples removed after 30, 60, and 90 days for the following: alcohol, extract, reducing

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TABLE 1

Effect of Various Treatments on the Composition and Characteristics of California-Type Sherry

Treatment	Alcohol				Aldehyde[b]			Color[c]				Transmission[d]		Total Neutral Esters[e]			Volatile Neutral Esters[f]		
	0 days	30 days	60 days	90 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days	30 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
	% by volume				mg. acetaldehyde/l.							%		mg. ethylacetate/l.			mg. ethylacetate/l.		
1. Untreated																			
a. Aerated	20.43	20.28	20.00	19.59	59.8	83.3	77.4	15.9	29.8	36.6	44.0	38.9	34.9	214	237	466	87	66	52
b. Unaerated	20.43	20.47	20.45	20.25	24.4	44.8	36.5	15.9	15.1	13.7	19.9	54.2	47.7	258	390	493	67	102	64
2. Calcium carbonate																			
a. Aerated	20.49	20.32	19.83	19.73	62.2	83.0	78.7	14.9	28.2	29.5	44.2	39.8	34.0	233	320	440	62	59	66
b. Unaerated	20.49	20.26	20.42	20.36	34.9	44.3	34.3	14.9	16.2	14.8	20.7	53.5	46.8	324	328	445	113	81	67
3. Dextrose																			
a. Aerated	20.15	20.09	19.76	19.30	65.2	83.3	85.0	14.8	29.1	28.6	42.8	39.2	34.3	249	352	483	81	38	47
b. Unaerated	20.15	20.28	20.26	19.93	31.8	43.0	41.2	14.8	16.6	14.6	22.3	52.7	47.0	309	399	473	97	102	59
4. Sulfur dioxide																			
a. Aerated	19.83	19.78	19.47	19.09	51.8	70.2	68.6	13.2	27.4	29.3	41.3	41.1	34.7	248	333	419	56	66	57
b. Unaerated	19.83	19.96	19.81	19.81	33.5	40.9	32.3	13.2	13.7	13.2	18.1	57.4	52.2	254	405	488	61	61	78
5. Citric acid																			
a. Aerated	20.24	20.21	19.80	19.36	64.7	81.8	80.0	14.2	24.1	21.8	35.9	44.5	38.5	319	358	548	102	70	51
b. Unaerated	20.24	20.51	20.12	20.06	35.1	43.2	40.8	14.2	14.0	13.7	20.4	56.3	47.6	314	410	542	107	91	77
6. Calcium carbonate + Dextrose																			
a. Aerated	20.21	20.02	19.71	19.44	64.8	83.3	88.0	14.8	32.3	31.5	46.4	36.0	32.4	258	294	394	118	53	58
b. Unaerated	20.21	20.37	20.34	20.05	33.1	40.8	38.3	14.8	15.9	13.9	22.8	53.4	45.7	273	338	492	81	67	73
7. Calcium Carbonate + Sulfur Dioxide																			
a. Aerated	20.30	20.19	19.83	19.50	61.3	81.3	85.5	15.1	31.1	28.6	48.5	37.1	32.0	268	335	456	107	57	45
b. Unaerated	20.30	20.35	20.44	20.26	34.3	40.1	31.5	15.1	13.2	12.2	17.6	58.3	53.1	299	363	474	92	62	64
8. Dextrose + Sulfur Dioxide																			
a. Aerated	20.24	19.97	19.64	19.05	55.7	73.3	76.3	13.9	30.2	28.4	42.7	38.3	31.4	258	370	451	102	72	58
b. Unaerated	20.24	20.25	20.20	20.04	35.4	42.1	33.2	13.9	13.2	11.7	19.6	57.2	52.1	293	359	514	61	72	77
9. Dextrose + Calcium Carbonate + Sulfur Dioxide																			
a. Aerated	19.17	19.00	18.68	18.31	57.4	77.9	66.5	13.3	29.8	31.4	47.3	37.8	31.7	278	319	429	107	51	55
b. Unaerated	19.17	19.29	19.31	19.13	35.0	38.4	31.3	13.3	13.4	11.8	17.2	58.1	53.2	249	338	.....	82	62	....
10. Averages																			
a. Aerated	20.12	19.98	19.64	19.26	60.3	79.7	78.4	14.5	29.1	28.9	43.7	39.2	33.8	258	324	454	91.3	59.1	54.3
b. Unaerated	20.12	20.19	20.15	19.99	33.1	42.0	35.5	14.5	14.6	13.3	19.8	55.7	49.5	286	370	490	84.6	77.8	69.9
c. Total	20.12	20.09	19.89	19.63	46.7	60.8	57.0	14.5	21.9	21.1	31.8	47.4	41.6	272	347	471	87.9	68.4	61.6

[b] Original was 10 mg./l. [c] Determined in a Duboscq colorimeter against a color standard. The color increases as the figures increase. [d] Original was 60.6%. [e] Original was 175 mg./l. [f] Original was 50 mg. /l.

TABLE 2

Effect of Various Treatments on the Composition and Characteristics of California-Type Sherry

Treatment	Extract				Sulfur Dioxide[a]		Total Acid				Volatile acid				pH			
	0 days	30 days	60 days	90 days	30 days	60 days	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days
	g. 100/g.				*mg./l.*		*g. tartaric/100 ml.*				*g. acetic/100 ml.*				*pH*			
1. Untreated																		
a. Aerated	2.6	2.6	2.5	2.4	0.0	0.0	0.55	0.52	0.53	0.52	0.077	0.062	0.063	0.061	3.60	3.68	.....	3.68
b. Unaerated	2.6	2.6	2.6	2.4	....	0.0	0.55	0.52	0.52	0.51	0.077	0.062	0.062	0.061	3.60	3.68	.....	3.70
2. Calcium Carbonate																		
a. Aerated	2.55	2.55	2.5	2.3	....	....	0.49	0.47	0.48	0.47	0.074	0.063	0.065	0.064	3.76	3.75	.....	3.80
b. Unaerated	2.55	2.55	2.4	2.3	4.9	....	0.49	0.47	0.48	0.45	0.074	0.063	0.064	0.061	3.76	3.78	.....	3.80
3. Dextrose																		
a. Aerated	4.1	3.9	3.9	4.1	1.3	....	0.55	0.52	0.53	0.52	0.064	0.062	0.061	0.060	3.62	3.67	.....	3.70
b. Unaerated	4.1	4.1	4.0	4.1	....	....	0.55	0.52	0.53	0.50	0.064	0.061	0.063	0.059	3.62	3.70	.....	3.70
4. Sulfur Dioxide																		
a. Aerated	2.5	2.6	2.6	2.6	46.0	0.0	0.56	0.55	0.55	0.52	0.077	0.063	0.063	0.060	3.60	3.62	3.60	3.61
b. Unaerated	2.5	2.6	2.5	2.4	36.5	0.0	0.56	0.54	0.54	0.51	0.077	0.064	0.066	0.062	3.60	3.64	3.69	3.64
5. Citric Acid																		
a. Aerated	2.55	2.7	2.8	2.8	2.0	....	0.73	0.69	0.68	0.67	0.068	0.063	0.061	0.063	3.48	3.52	3.51	3.58
b. Unaerated	2.55	2.7	2.7	2.5	....	....	0.73	0.67	0.67	0.66	0.068	0.063	0.063	0.062	3.48	3.52	3.50	3.55
6. Calcium Carbonate + Dextrose																		
a. Aerated	3.9	3.9	3.9	3.8	....	....	0.47	0.46	0.47	0.46	0.066	0.063	0.064	0.064	3.80	3.80	3.80	3.79
b. Unaerated	3.9	3.9	3.95	....	5.3	....	0.47	0.47	0.47	0.44	0.066	0.061	0.065	0.062	3.80	3.81	3.79	3.85
7. Calcium Carbonate + Sulfur Dioxide																		
a. Aerated	2.5?	2.5	2.5	2.5	....	....	0.49	0.47	0.48	0.48	0.068	0.064	0.062	0.065	3.75	3.75	3.77	3.80
b. Unaerated	2.5?	2.5	2.5	2.4	37.1	....	0.45	0.47	0.47	0.46	0.068	0.064	0.066	0.062	3.75	3.80	3.78	3.80
8. Dextrose + Sulfur Dioxide																		
a. Aerated	3.9	3.9	4.0	4.05	40.0	....	0.56	0.53	0.54	0.53	0.071	0.061	0.063	0.064	3.60	3.61	3.61	3.61
b. Unaerated	3.9	3.95	4.0	4.0	24.2	0.0	0.56	0.51	0.52	0.52	0.071	0.064	0.064	0.069	3.60	3.65	3.63	3.68
9. Dextrose + Calcium Carbonate + Sulfur Dioxide																		
a. Aerated	3.95	3.9	4.0	3.8	37.8	0.0	0.48	0.47	0.48	0.46	0.074	0.063	0.063	0.061	3.71	3.73	3.75	3.79
b. Unaerated	3.95	3.9	4.0	....	....	0.0	0.48	0.47	0.48	0.46	0.074	0.066	0.067	0.065	3.71	3.79	3.75	3.80
10. Averages																		
a. Aerated	3.2	3.1	3.2	3.1	....	....	0.54	0.52	0.53	0.51	0.071	0.063	0.063	0.063	3.66	3.68	3.67	3.71
b. Unaerated	3.2	3.1	3.2	2.9	....	....	0.54	0.52	0.51	0.51	0.071	0.063	0.065	0.063	3.66	3.70	3.69	3.72
c. Total	3.2	3.1	3.2	3.0	....	....	0.54	0.52	0.52	0.51	0.071	0.063	0.064	0.063	3.66	3.69	3.68	3.72

[a] Original was 100 in samples to which sulfur dioxide was added.

sugar, total acid, volatile acid, pH, sulfur dioxide, aldehyde, total neutral esters, volatile neutral esters, tannin, color, percent transmission, and hydroxymethylfurfural. The regular methods of analyses, used in this laboratory, as outlined by Amerine *(1)* were employed except for percent transmission, which was determined in using an 8 mm. cell in a Coleman photoelectric spectrophotometer set at 400 mμ. The results are given in Tables 1 and 2.

The analysis of variance procedures of Brownlee *(3)* and Fisher *(4)* were applied to the data. There were nine lots of wine each treated in two ways, aerated and unaerated. Since comparisons were available at 30, 60, and 90 days, a total of 54 comparisons could be made. A simple analysis of variance for the significance of treatment, time, or aeration can be made only if there are no interactions between these factors. In order to illustrate the methods used the analysis for alcohol is given in detail.

RESULTS

The original alcohol content in the untreated lot was 20.43%. Slight differences between the lots were found, particularly where sulfur dioxide had been added. During heating there were average actual losses of 0.85% from the aerated group and of 0.13% from the unaerated group. Since there was little or no loss by vaporization from the latter, the 0.13% loss may represent changes due to chemical reactions such as esterification and aldehyde formation. The interactions, treatment x time and treatment x air, were not significant when all of the data were analyzed together. Time x air was significant. When all the data were considered, taking into account the time x air interaction, the "F" values for time and air were not significant, but those for treatment were. Since inspection showed a difference between the aerated and unaerated samples the interaction had probably prevented the "F" values from reaching significance. The data were then separately analyzed for aerated samples and for unaerated samples. The analysis of variance data are given in Table 3. In both cases,

TABLE 3

Analysis of Variance for Alcohol

Source	D.F.[b]	Sum of Squares	Variance	F	$F_{0.05}$	$F_{0.01}$
Aerated						
Total	26	6.25	.....	.....	.....	.....
Treatment	8	3.38	0.479	95.8	2.59	3.89
Time	2	2.34	1.170	234.0	3.63	6.23
Time x treatment	16	0.08	0.005	.....	.....	.....
Unaerated						
Total	26	3.51	.....	.....	.....	.....
Treatment	8	3.14	0.392	39.2	2.59	3.89
Time	2	0.21	0.105	10.5	3.63	6.23
Time x treatment	16	0.16	0.010	.....	.....	.....

[b] Degrees of freedom.

differences due to treatment and to time were highly significant. Differences in time seemed to be more important for the aerated than for the unaerated series.

Student's t-test when applied to the difference between the means of the aerated series, 19.63, and the unaerated series, 20.11, gives for t the value 20 with 32 degrees of freedom. The variance used, 0.0075, is the average

TABLE 4

Factorial Analyses of Effect of Treatment on Alcohol Changes in California-Type Sherry

Treatment	Total	Effect of						
		$CaCO_3$	Dextrose	SO_2	$CaCO_3$ and Dextrose	$CaCO_3$ and SO_2	Dextrose and SO_2	$CaCO_3$, Dextrose, and SO_2
Untreated	121.04	−0.12	−1.42	−3.12	0.43	2.77	2.65	−8.51
$CaCO_3$	120.92	.....	−0.99	−0.35	8.08	−5.74	−5.86	.....
Dextrose	119.62	0.31	.....	−0.47	.....	.....	.....	.....
$CaCO_3$ and Dextrose	119.93	.....	.....	−6.21	.....	.....	.....	.....
SO_2	117.92	2.65	1.23	.....	.....	.....	.....	.....
$CaCO_3$ and SO_2	120.57	.....	−6.85	.....	.....	.....	.....	.....
Dextrose and SO_2	119.15	−5.43	.....	.....	.....	.....	.....	.....
$CaCO_3$, Dext., and SO_2	113.72	.....	.....	.....	.....	.....	.....	.....

of the values in Table 3. This is a highly significant result which indicates that aeration reduced the average alcohol content.

Except for citric acid all of the treatments formed a factorial arrangement as indicated in Table 4. At the 5% level the least significant difference of totals of 24 was 1.26. Thus the second order interaction was significant. This means that the effect of one substance was dependent on the presence or absence of the others. Calcium carbonate by itself or with dextrose had little or no effect. In the presence of sulfur dioxide it had an increasing effect. Dextrose alone or with calcium carbonate had a depressing effect while in the presence of sulfur dioxide it had an increasing effect. Sulfur dioxide alone had a pronounced negative effect. In the presence of either dextrose or calcium carbonate it had little or no effect. Each of these substances in the presence of both the others had a pronounced depressing effect.

The increases in alcohol noted above were not actual increases but simply reflected the reduction in losses that occurred with time. This analysis indicated that calcium carbonate in the presence of sulfur dioxide prevents loss of alcohol. It is unlikely that the effect was an analytical one. The depressing effect of sulfur dioxide alone or in the presence of dextrose may have been due to some effect of sulfur dioxide on the accuracy of the analytical procedure for alcohol.

The original aldehyde content was 10 p.p.m. which is very low. During heating aldehydes markedly increased in all samples, but the aerated group developed approximately twice as much as the unaerated. The greater portion of the increase occurred during the first 60 days. Analyses of all the data revealed that the interactions, treatment x air and time x air, were significant. The data for the aerated and unaerated samples were therefore analyzed separately. The calculated "F" values for treatment and for time for the aerated samples were both highly significant. For the unaerated samples, treatment was not significant but time was. The significance of time was obviously due to the cumulative oxidation of alco-

TABLE 5

Effect of Treatment on Aldehyde Changes in Aerated Samples of California-Type Sherry

Treatment	Total	$CaCO_3$	Dextrose	SO_2	$CaCO_3$ and Dextrose	$CaCO_3$ and SO_2	Dextrose and SO_2	$CaCO_3$, Dextrose, and SO_2
Untreated	22.05	0.34	1.30	−2.99	−0.08	4.21	0.17	−4.82
$CaCO_3$	22.39	.....	.....	.....	−4.90	−0.61	−4.65	.....
Dextrose	23.35	0.26	1.47	−1.22	.....	.....	.....	.....
$CaCO_3$ and Dextrose	23.61	.....	.....	.....	.....	.....	.....	.....
SO_2	19.06	4.55	1.22	−2.82	.....	.....	.....	.....
$CaCO_3$ and SO_2	23.61	.....	.....	.....	.....	.....	.....	.....
Dextrose and SO_2	20.53	−0.35	−3.43	−3.43	.....	.....	.....	.....
$CaCO_3$, Dext., and SO_2	20.18	.....	.....	.....	.....	.....	.....	.....

hol to aldehyde. Since only the aerated samples showed a significant effect of treatment, the factorial analysis was applied only to these data. The results are given in Table 5. The least significant difference between the totals of 12 items was 3.09. Hence, the interactions were significant. Sulfur dioxide had a uniformly depressing effect on the formation of aldehydes. This was to be expected because of its anti-oxidant properties and may indicate that oxygen was the limiting factor in the formation of aldehyde, even though little or no sulfur dioxide was present in the wines after 60 days. Calcium carbonate had little effect except in the presence of sulfur dioxide and then it had a pronounced, increasing effect. This result is puzzling. Dextrose had a uniformly increasing effect except in the presence of both calcium carbonate and sulfur dioxide when it had a depressing effect. These results, again, cannot be explained with our present knowledge.

The difference between means of treatments may also be tested by the use of "t" values calculated from the analysis of variance data. The totals for all aerated samples for 30, 60, and 90 days were 54.29, 71.74, and 70.60, respectively. Similarly, the totals for unaerated samples were 29.75, 37.76, and 31.94. The least significant difference of totals of nine items at the 5% level was 2.73. Thus, all samples increased during the first 60 days and then decreased during the 60 to 90 day period. The decrease for aerated samples may not be statistically significant.

Color was one of the first differences noted. The lot containing only sulfur dioxide was appreciably lighter. However, other lots containing sulfur dioxide and dextrose did not always show a reduced color. Generally, color decreased in the unaerated samples during the first 60 days of heating. This may have resulted from the difficulty in filtering the original wine to a completely brilliant state. Possibly heating precipitated some of the suspended material and the samples after 30 to 60 days became less cloudy. The final color of the unaerated samples was greater than that of the original wine. The color of the aerated group increased regularly throughout the heating, and the final color was approximately twice

as great as that of the unaerated group and three times that of the original wine.

Since the interactions, treatment x time and time x air, in this case were both significant, in order to analyze the data, the 30, 60, and 90 day intervals were considered separately. At 30, 60, and 90 days, the influence of treatments was insignificant on the development of color, while the aerated versus unaerated samples showed a highly significant difference ("F" calculated values of 274, 209, and 239 compared to the theoretical of 6.03 for significance at the 1% level). Differences with time, calculated from the means at 30 and 90 days, and 60 and 90 days were highly significant but for the 30 and 60 day means they were not.

Similar changes in percent transmission at 400 mμ were also found; the aerated group decreased in percent transmission much more rapidly than in the unaerated group. Slight differences between various treatments were noted. The analysis of variance for transmittance showed only the interaction treatment x time to be significant. The data were analyzed altogether, and the influence of treatments was not found to be significant. Both time and air had a significant effect. These data were also analyzed at 30 and 90 days, taking into account the significant interaction of treatment x time. Again treatment was not significant but aeration was highly significant. The appropriate "t" value for comparing transmittance at the end of 30 and 90 days was 4.64. This value was highly significant and indicated that time influenced transmittance.

The total neutral ester content at the start was 175 mg. per liter as ethyl acetate. This increased in all samples during heating, the final values being about 475 mg. per liter. The citric-acid-treated sample, lot 5, had the largest ester content. The volatile neutral ester content in the original wine was 50 mg. per liter as ethyl acetate. The volatile esters increased during the first 30 days of heating. During the last 60 days, little change was noted in the unaerated group, but a marked decrease was evident in the aerated group. At 65.5°C.(120°F.) the vapor pressure of ethyl acetate is appreciable, hence the air space above the wine in the two-gallon jugs was relatively rich in volatile esters. As air was drawn through these samples at weekly intervals, some of this ester-rich vapor was probably removed. The analysis of variance for total neutral esters and volatile neutral esters was similar. None of the interactions was significant; and neither treatment nor aeration had a significant effect. Time, however, was highly significant in its effect in both cases.

The changes in the extract value during heating were small, though the tendency was for it to decrease. The samples to which dextrose had been added naturally had higher extract values than the others. Analysis of variance indicated that only differences between means of the treatments were significant, obviously due to the dextrose added to lots 3, 6, 8, and 9.

Total sulfur dioxide was determined on only a limited number of samples. After 30 days little or none was present in the samples to which no sulfur dioxide had been added. In the sulfur dioxide-treated samples, only traces were found after 60 days.

The total acidity decreased slightly during heating in both the aerated and unaerated groups for all treatments. Analysis of variance, indicated that treatment, time, and aeration all had significant effects and that the treatment x time interaction was important. When a factorial arrangement of the data (omitting citric acid) was made, only the self-obvious calcium carbonate treatment showed a significant effect. Volatile acidity decreased slightly during heating with no differences being noted between the aerated and unaerated samples. No very significant effect was indicated by analysis of variance, the differences being small with respect to the unit of measurement. The treatment x air interaction might be significant. Using it, the treatment and air effects were not significant. The pH increased in some but not all samples; the change, however, was very small, being less than 0.1 pH unit.

Tannins were determined on only a few samples (not reported in the tables). The original tannin content decreased from 0.019% to 0.013% for the unaerated samples and to 0.010% for the aerated. A brown deposit was found on the bottom of all containers. The volume of this deposit appeared to be slightly greater in the aerated than in the unaerated samples.

The amount of hydroxymethylfurfural, detected by Fiehe's test in all samples after 30 days of heating, was very slight, as would be expected in low levulose wines.

Oxidation-reduction potentials were determined on a Beckman pH meter, using a clean platinum electrode, after 30 days of heating. A higher potential was found in the aerated samples, but no regular differences between treatments were observed. The results are not reported here because of the drifting potentials encountered, even when air was rigorously excluded during sampling and measurement.

Standard triangular taste tests, according to Amerine *(2)*, were made on the aerated and the unaerated wines about 60 days after the conclusion of the experiment. During the interim period, the wines had been stored in glass containers at 12.8°C.(55°F.). Six tasters made a total of 42 tastings. The odd sample was correctly identified in 31 cases. According to Roessler, Warren, and Guymon *(6)* this is highly significant that a real difference in taste exists between wines treated by the two methods. During the same test the tasters were asked to state their preference between the two treatments. No clear preference for either sample was noted in the 31 correct identifications, some tasters preferring one wine at one time but not at another. These tests were based on only smell and taste, as the test conditions (by the use of glasses painted black) prevented observation of the obvious color differences between the aerated and unaerated samples. Generally the aerated samples seemed to have a stronger "burnt" or California-type sherry odor, while the unaerated samples appeared to be more ethereal and less distinctive in odor. About half of the tasters found the odor of the unaerated samples to be more pleasant.

SUMMARY AND CONCLUSIONS

1. Aeration of shermat during heating led to losses of alcohol of nearly 1%. The decrease in alcohol in the unaerated samples amounted to less

than 0.20%. The statistical analyses of the data indicated that the loss was due to the treatments and that the difference did not, under the conditions of this experiment, become significant until 90 days.

2. Aldehydes likewise increased during the first 60 days of heating and thereafter decreased. The sulfur dioxide-treated samples were significantly lower in aldehydes in 3 of 4 samples. Much greater quantities of aldehydes were produced in the aerated than in the unaerated samples.

3. In the aerated samples, there was an immediate increase in color, followed by a slight but insignificant drop and an increase during the final 30 days. Only slight, but again insignificant, changes in color were noted in the unaerated samples for 60 days, but during the 60 to 90 day period, a marked increase occurred. With the exception of the sulfur dioxide-treated lot, the citric acid-treated sample was significantly lower in color than the other lots. With regard to percent transmittance, only the decrease with time and between the aerated and unaerated samples was found to be significant.

4. Total neutral esters increased rapidly with time, but no significance between treatments was noted. Volatile neutral esters increased markedly at first and decreased thereafter.

5. Analysis of variance was applied to all the data to determine the significance of the various treatments, of time, and of aeration.

In these experiments the effect of treatments on alcohol changes are a useful indication for further experimentation.

In general, it may be concluded that aeration and length of heating have had the greatest influence on the changes in composition in this experiment. Differences in the composition of the wine being heated did not result in any marked difference in color or composition with the exceptions noted above.

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CHICKEN FLAVOR: THE SOURCE OF THE MEAT FLAVOR COMPONENT

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In the first paper of this series *(1)*, the method of separating the chicken flavor from a broth was described. The second paper *(2)* went on to describe the properties of the flavoring extracts and several methods for the fractionation of the separate components.

During the fractionation of the chicken broth it became evident that there was a separation of the extract into two flavors: one was a sulfur-containing fraction which was described as meaty since its odor was not typically chicken flavor; the other was that portion of the flavor which definitely contributed the characteristic chicken odor (or flavor) and for that reason will be called the "character fraction" in subsequent discussions.

The present paper deals with the source of the "meat" flavor, with reference first to tissues concerned, and second to the chemical constituents of these tissues which contribute the meat flavor.

The chicken flavor is produced during the cooking process, for when raw chicken is eaten there is no recognizable flavor. If the raw meat, which has been chewed for some time, is returned to the cook pot, a definite odor of cooked chicken is evident. It seems, therefore that the flavor is a reaction product of the heat upon the tissues.

The first problem was to establish which tissues contribute the meat flavor, since the properties of the character flavor are best studied in the presence of the meaty component. The simplest division of tissue in the animal body is the protein from fat. The first experiments were conducted to determine the importance of fat in the meaty flavor.

In the meat processing industry great importance is placed on the fat for its flavoring properties. Care is taken to include some fat in every canned meat product. Some fat is usually found in most types of canned soups, it is even found in dehydrated soup preparations. How important is the fat in the establishment of the meat flavor?

EXPERIMENTAL

A large mature white leghorn cockerel was slaughtered and the fat from within the body cavity removed and separated from the organs. This fat was cooked for twenty minutes on a steam bath, under total reflux, then steam was applied directly into the fat to distill any flavoring material into a connected receiver. Upon acidification of the distillate, only a trace of flavor was evident, and this may have been due to that small amount of fibrous tissue which encased the leaf fat. The leaf fat contributes very little to the chicken flavor.

A second animal was slaughtered and the leaf fat removed and discarded. The eviscerated carcass was completely homogenized in absolute alcohol, then dried bone-dry in an oven. The dry material was finely ground and again dried in a vacuum

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desiccator. The ground, dry chicken was then extracted with pure iso-pentane in a Soxhlet extractor for six hours. The solvent was evaporated from both the fat and tissue by slight warming. The fat was placed in the reflux and cooked for twenty minutes; then steam was applied as before. The distillate had a fatty-acid-like odor. No meat flavor was evident. A similar extraction experiment was conducted using pure acetone, a much more polar solvent, with similar results. However, the extracted meat, when cooked with water, did produce a meaty flavor. Thus it appears that the flavor component of meat is not in the fat. It is more closely associated with the meat fibers.

A problem persists in relation to the fat and chicken flavor; for when the fat is skimmed from a broth rich in flavor, it is quite strongly flavored even when there is no water present. Perhaps the flavor, once produced, is soluble in fat.

The Separation of the Meat Flavor from the Fibrous Tissue. Since it has been established that the tissue fibers or associated substances are responsible for the meat component, it seemed quite important to study the fibers themselves to see what particular substance contributes this flavor.

Crocker *(3)*, in a study of the meat flavor, found he could squeeze raw meat free of its juices, and stated that the press juice contained no flavor when cooked. The fibers maintained the ability to produce flavor when cooked even after repeated squeezes. The author *(1)* has demonstrated that the flavor of chicken could be cooked into water to form a broth. The broth contained the true chicken flavor and the meat was completely extracted of all its flavor after cooking.

An experiment was conducted to determine whether the protein itself or some substance associated with the protein in the animal fibers was responsible for the meat flavor. A whole chicken was homogenized in 5% trichloracetic acid (TCA). The solids were filtered off with gentle suction, and again suspended in the TCA. The protein was washed in this manner five times and then dried under vacuum after repeated washings with ethyl ether. When this tissue was boiled in water no flavor was evident. Thus the flavor is extractable from the protein.

A second experiment to determine whether the flavor is extractable from the fibrous material was conducted. A 50-gram sample of the meat which had been dried and extracted with solvent was extracted with ten separate 100-c.c. portions of 60% methyl alcohol. The tissue was dried in a vacuum desiccator and tested for flavor retention by cooking in water. All of the flavoring properties had been removed by the extraction with the alcohol solution. Here again it seems that the flavor is not derived from the protein The flavor of chicken meat is derived from a substance which is not associated with the fat, but is attached to the fibers and cannot be removed by pressing. It is a soluble substance which is extractable from the meat with dilute TCA or 60% methyl alcohol. It therefore must be a compound of relatively low molecular weight.

GLUTATHIONE AND THE MEAT FLAVOR

In the preceding chapters, Bouthilet *(1, 2)*, it was shown that the meat-flavoring compound was either a sulfur compound or a breakdown product of some sulfur-containing compound found in the animal body. During distillation of the flavor, ammonia was evolved because, as the distillate grew progressively alkaline, an ammonia derivative was produced.

Crocker has pointed out and the author has stressed the fact that hydrogen sulfide is produced during the cooking of meat or poultry. It is produced from the extract by any manipulation, such as agitation or bubbling of air, which might involve oxidation.

The following points are known concerning the source of the flavor: (1) The flavor exists as an integral portion of the meat fibers; (2) it can be extracted from the fibers by prolonged steeping of the meat in water; (3)desulfuration accompanies the production of the flavor; (4) ammonia is produced during distillation (or oxidation) of the flavor; (5) the compound is a breakdown product of another sulfur-containing normally occurring material.

This cursory information led us to search the literature for likely compounds that might be responsible for the meat flavor. Proteins were eliminated because of their stability to heat (60°C.). Because of their relative stability, amino acids and carbohydrates, except for glutamic acid and proline which become volatile compounds under slight stress, were also eliminated. Whole fats were eliminated by the preliminary experimental work reported here.

The following sulfur-containing compounds were suspected of contributing to the flavor: (1) Ergothionine, (2) the thiolactone of homocysteine, (3) glutathione, and (4) some unknown compound in the meat. Of these, glutathione is the most unstable and is easily available, and for this reason it was investigated thoroughly.

Hopkins *(4)* discovered glutathione, and reported it as a dipeptide of glutamic acid and cysteine. Eight years later, after it was discovered that glycine was also a portion of the compound, he made a reinvestigation, Hopkins *(5)*. Hopkins' study of glutathione was extensive; he studied the properties of the compound under all conditions. He was particularly insistent on the purity of his product. From his discussion it would appear that purified preparations were more unstable than the crude. Such a property would be expected of glutathione if some natural antioxidant were present which prevented decomposition of crude extracts.

In his discussion of the properties of glutathione, Hopkins says: "On boiling with water desulfuration occurs from the first and the evolution of H_2S is continuous throughout such treatment." In a discussion of loss of N and S in the oxidation of the very pure product Hopkins goes on (p. 276) to say: "The nitrogen must have been removed as ammonia and the sulfur as sulfuric acid or in some volatile form." He later states that the glutamic acid produced in the decomposition with heat is found chiefly in the pyrrolidone carboxylic acid form.

The following points seem indicative of a relation of glutathione to the flavoring material: (1) Desulfuration, (2) ammonia formation, and (3) pyrrolidone carboxylic acid formation. In the last analysis the best presumptive test for a flavor is taste and odor. A sample of glutathione, Eastman, was tasted and smelled after heating in water. It smelled like meat. When a little sodium hydroxide was added to dispel the acid it also tasted like meat. Another sample was prepared from yeast by the method described in Morrow and Sandstrom *(6)*. It, too, tasted like meat. A third sample was made from chicken flesh and it also tasted like meat.

A discrepancy exists in the flavor of glutathione and its comparison with meat. Whenever glutathione is tested for homogeneity by paper-partition chromatography, five spots or more are found which show in ultra-violet light. Several of these show with ninhydrin or sulfur reagent. Therefore, one cannot be sure whether the meat flavor is glutathione or an impurity in the sample of glutathione. It might well be an impurity, since it takes only a small amount of the flavor to give a strong odor.

It is also entirely possible that the meat flavor is a breakdown product of glutathione. The most tasty meat preparations are those which are cooked at low temperatures. These conditions would be proper for the

breakdown of glutathione without the distillation of the volatile flavor. It is a well-known fact that large cuts of meat give more flavor than small ones; this could also be due to the inhibition of oxidation until the meat is chewed.

The glutamic acid produced from the glutathione might well be that portion which is related to the monosodium glutamate as a flavoring material.

SUMMARY

The "meaty flavor" in chicken meat is due to a compound associated with the meat fibers rather than the fat. It can, however, be extracted from the fibers by steeping in water or by extraction of the meat after suitable denaturation of the protein.

When the properties of the water-soluble flavoring compound are compared with those of known chemical compounds, it appears that the flavoring precursor has properties similar to glutathione. Samples of pure glutathione have an odor similar to meat when dissolved in water and heated to 60°C. The taste is similar to meat when the acid is neutralized.

Since there is some doubt as to the purity of the available glutathione, the relation between the meat flavor and glutathione still awaits identification of all the isolated meat-flavoring components and their comparison to the heat-induced breakdown products of glutathione.

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AN EXPERIMENTAL APPROACH TO PROBLEMS OF CONTROL IN QUANTITATIVE STUDIES OF ULTRASONIC BACTERICIDAL EFFECTS

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The results of previous ultrasonic studies on bacterial suspensions have yielded valuable qualitative information, but in most cases one or more of the quantitative factors remained uncontrolled. In order to proceed from a qualitative to a quantitative investigation it was necessary to establish, from the fundamental principles of acoustics and the experimental studies of previous investigators, the controls required. Since a comprehensive analysis of this problem has not been reported previously, the literature has been studied with that purpose in mind and the controls shown to be necessary for quantitative study have been undertaken experimentally. It is hoped that this presentation of the problems involved will be of special value to those anticipating the use of ultrasonics in their food research.

LITERATURE

Energy Distribution. Of primary importance in the quantitative definition of a system under ultrasonic radiation is the factor of energy distribution, which has been investigated most completely by Boyle and co-workers *(3, 4, etc.)*. They found *(3)* that the greater portion of the energy generated was concentrated in a central diffraction beam arising at a distance from the crystal determined by its radius and frequency and having an angular spread which was also a function of these quantities.

Wood and Loomis *(14)* showed that when the distance between the vibrating source and a reflecting barrier, such as the floor of the radiation chamber, was an integral number of half-wavelengths, the intensity of radiation was a maximum. However, Smith and Stumpf *(11)* suggest that this potential increase in intensity be sacrificed to prevent the coupling which results and alters the natural frequency of the crystal. With a perfectly transmitting floor, standing waves between the source and the floor of the chamber would be almost completely eliminated, but reflection from the surface of the liquid (approximately 100%) in the chamber would result in maximum coupling if the distance from the source to the surface were an integral number of half-wavelengths.

The thickness of the floor of the radiation chamber was shown by Boyle and Rawlinson *(4)* to be critical in regulation of the amount of energy transmitted to the suspension. When the thickness of the plate is an integral number of half-wavelengths, the amount of energy transmitted

is independent of the other characteristics of the plate and is dependent only upon the acoustical impedance of the two media. When these values are equal, all the energy is transmitted if there are no energy losses in the plate. At all other thicknesses, the transmitted energy is a function of the acoustical impedance of the plate as well as of those of the two media. As long as the former is large compared to the square root of the product of the latter, which is the case of all commonly used plate materials and media, the amount of energy transmitted is a minimum when the thickness of the barrier is an odd integral number of quarter-wavelengths. Therefore, for maximum transmission of energy through the barrier, its thickness should be an integral number of half-wavelengths.

Not only is the floor of the radiation chamber set in vibration by the energy striking it but, as shown by Giebe and Blechschmidt *(6)*, the walls of the tube, if exposed to high intensity radiation, are subject to coupled transverse and longitudinal vibrations. To allow maximum energy transmission into the sample being irradiated, it is desirable, therefore, to minimize the passage of energy into the walls of the tube and the potential interference of the resultant wave trains.

Temperature Control. The heat generated by ultrasonic radiation makes temperature control of paramount importance in the irradiation of bacteriological systems, Harvey and Loomis *(8)*. Two major considerations are involved:

1. The dimensions of the crystal, which control its natural frequency, and the wavelength of sound in the oil are functions of temperature. The constancy of frequencies and distance relationships expressed in terms of wavelength is therefore dependent upon temperature control.
2. Uncontrolled addition of heat to a bacterial suspension obscures the interpretation of the effects of ultrasonic radiation.

Temperature control becomes increasingly difficult as the frequency is raised because the conversion of ultrasonic energy into heat increases with the frequency. Thus, the studies in which temperature control was readily attained are either cases of low-frequency radiation or of very low-intensity radiation. The presence of an air-liquid boundary at the surface of the suspension reinforces these heat sources by reflection of the ultrasonic energy.

Bacteriological Control. The indications of the desirability of bacteriological control are numerous in the literature. Factors such as the type of organism, its age, its initial population in the suspension under irradiation, the medium in which it is suspended, and the method used to determine the population seem to require definite consideration. The observations of Yen and Liu *(15)* and Beckwith and Weaver *(2)* indicate the types of organisms vary considerably in their resistance to ultrasonic radiation with the spore formers the most resistant, coccus forms intermediate, and non-spore forming rods the least. Reports of the importance of the age of the culture are in less agreement. Chambers and Gaines *(5)*, Hamre *(7)*, and Williams and Gaines *(13)* found the rates of bacterial destruction to be independent of age under certain experimental conditions. However, Chambers and

Gaines *(5)*, working in the sonic range, noted considerable deviation from the logarithmic destruction of 12-48 hr. cultures of *Escherichia coli* in cultures five or more days old. The importance of the initial population is not clear though Dorothy Hamre *(7)* noticed a slight increase in the rate of destruction when the count of a suspension of *Klebsiella pneumoniae* was increased from 3×10^5 to 9×10^7. Little study has been devoted to the effect of the bacterial substrate, but Dorothy Hamre *(7)* found a higher rate of destruction of *Klebsiella pneumoniae* in broth than in 0.85% saline or in phosphate buffer, until the surface tension of the saline was lowered to that of the broth. Beckwith and Weaver *(2)* found the rate of destruction of *Escherichia coli* was less in the presence of gelatin than in the presence of sugar or fat. This may have been due to the higher viscosity of the gelatin. Stumpf, Green, and Smith *(12)* found that destruction of bacteria was curtailed markedly in a thick suspension of viscosity above a critical value and was increased greatly by sufficient dilution. Szu-Chih and Yen *(9)* observed a greater population decrease on irradiation when their suspension was saturated with air than when saturated with hydrogen. The importance of the method of determining the degree of destruction was demonstrated by the work of Harvey and Loomis *(8)* on luminous bacteria, in which the growth on plates made from the irradiated suspension was retarded as well as reduced.

EXPERIMENTAL

From the foregoing considerations it is clear that any approach to a study of the quantitative effects of ultrasonic radiation in which one of the commercially available piezoelectric types of generators is used poses the three major problems of 1. actual measurement or control and reproducibility of intensity, 2. temperature control within required limits for required lengths of time, and 3. bacteriological control from the stock culture through the incubation of plates after irradiation.

The ultrasonic generator used in the present work is quoted to have a maximum power output of 500 W radio-frequency energy equivalent to approximately 50-60 W acoustical energy per cm.^{2} on the surface of the crystal. The power source is separately housed in a metal cabinet and connected by coaxial cable to the transducer unit shown in Figure 1. Four frequencies, from 400-1,500 k.p.s. are supplied by four transducer units, each of which, when in use, becomes automatically connected to the proper crystal frequency-control within the generator. The power input is controlled by the power-stat knob on front panel of the generator, and front panel meters register the input current and potential. An automatic timer can be used to shut off the power after 5 minutes of radiation and, when reset, to instantly reestablish the power at the level of the previous radiation period.

Intensity Control. Hamre *(7)* and Shropshire *(10)* have presented data attempting to correlate intensity with bactericidal effect. In this work power input is taken as the measure of intensity. Such an assumption is by no means rigorous. The only true measure of intensity would be obtained by a probe directly measuring the intensity over a succession of areas covering the path of radiation throughout the bacterial suspension. However, at the initiation of this study, research on intensity gauges was in progress in the Department of Electrical Engineering at the University of Illinois. This made it seem expedient to concentrate on the control and reproducibility of the intensity through the control of physical factors such as chamber design and mounting and power input until the actual measurement of intensity became feasible.

A radiation chamber was designed to be permanent, readily sterilized, simple to construct, and resistant to corrosion, staining, etc. In accordance with the findings of Boyle, it was given a plane bottom for perpendicular incidence of the beam, a sufficient diameter to cover the central portion of the beam where the intensity is greatest and

to extend beyond the edge of the crystal sufficiently to prevent transverse vibrations being set up in the walls of the chamber, and a series of interchangeable floor plates of half- and quarter-wavelength thickness for maximum transmission and reflection respectively. The design and dimensions used in construction are shown in Figure 2. A cap nut ground to a tolerance of $\pm$ 1°, threaded to fit the tube, and cushioned by gaskets on each side of the plate, provides for perpendicular junction of plate and tubing, and for ease of assembling, dissembling, and cleaning and makes possible a water-tight seal. The choice of two materials in construction was based upon the

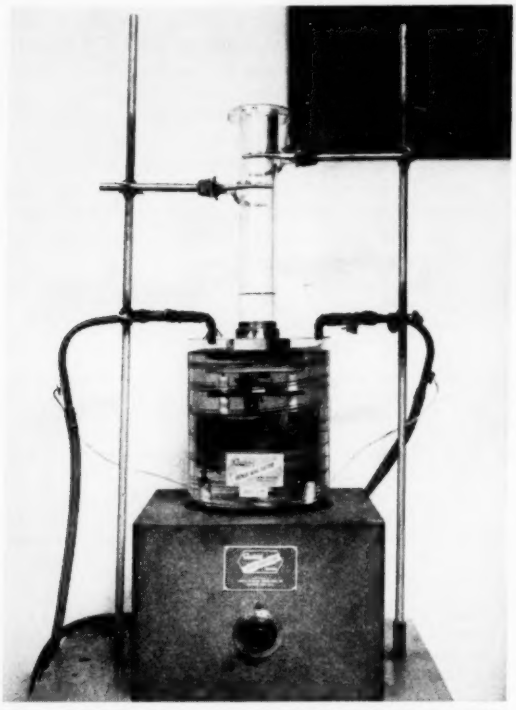

FIG. 1. Ultrasonic transducer and radiation chamber assembly.

desirability of studying the effect of the material of the chamber on the rate of destruction. Stainless steel 18-8 S Type 304 was selected for its high corrosive resistance, for ease of machining and unbreakability. Good quality borosilicate crown glass containing no macro reflecting surfaces was selected for the advantage of visual observation of radiation phenomena. In this case, however, it seemed more practicable to use a steel ferrule cemented to the glass tube and fitted with a steel nut. The construction was such that glass plates fit steel tubes and vice versa.

The dimensions of half- and quarter-wavelength plates for all four frequencies in both glass and steel were calculated from the velocity of sound in a solid plate of each material considered as a very short circular rod. The actual values of the elasticity, Poisson's ratio and Young's Modulus, and the density of the samples of glass and steel from which these plates were cut were used in the calculation of velocity, and the amount

of uncertainty in each value was taken into consideration.[a] A tolerance in the thickness dimensions for machining purposes was also calculated on the basis of a minimum of 90% transmission by the use of Boyle's expression for the percentage transmitted. The actual plate thickness specified and attained for 1,000 k.p.s., the frequency employed in this study, are given in Table 1.

TABLE 1

Plate Thicknesses for 1,000 k.p.s.

Glass			
Plate thickness, theoretical, cm.	Tolerance, cm. ($\pm$ 0.06812 λ)	Possible error, cm.	Plate thickness, actual, cm.
$\lambda/2 = 0.218$	$\pm$ 0.030	$\mp$ 0.005	0.218
$\lambda/4 = 0.109$	$\pm$ 0.030	$\mp$ 0.003	0.105
Steel			
Plate thickness, theoretical, cm.	Tolerance, cm. ($\pm$ 0.03632 λ)	Possible error, cm.	Plate thickness, actual, cm.
$\lambda/2 = 0.280$	$\pm$ 0.020	.....	0.277
$\lambda/4 = 0.140$	$\pm$ 0.020	.....	0.151

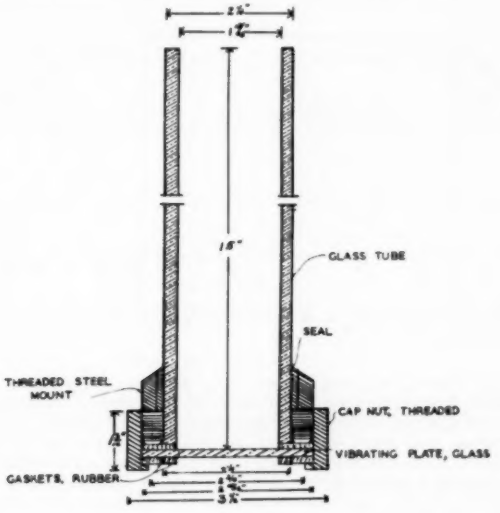

FIG. 2. Ultrasonic radiation chamber.

The chamber was mounted as shown in Figure 1, visually centered, with the floor plate parallel to the crystal as judged by a catchetometer, which was also used to measure the distance from the crystal to the bottom of the chamber and to the top surfaces of the suspension. Calculation indicated that the minimum achievable distance between crystal and bottom of the chamber was sufficient for utilizing the central diffraction beam.

[a] The steel employed and its constants were supplied by Chicago Steel Service Company. The glass plates were secured from Corning Glass Works. Dr. H. R. Lillie of their Physical Laboratory determined the physical constants.

Temperature Control. To control the temperature of the bath, the entire volume of oil was circulated through cooled stainless steel coils, as suggested by the work of Beckwith and Weaver *(2)*, Hamre *(7)*, Harvey and Loomis *(8)*, and Stumpf, Green, and Smith *(12)*. When this proved impractical because of the small temperature differential which could be supplied and because of operating difficulties encountered in the circulation of the oil with the pump available, the circulation of ice water through an 8-turn glass coil 7½" in diameter placed in the oil bath was tried. By this means the temperature could be lowered to 8-9°C. prior to radiation and did not rise above 38°C. during radiation.

Bacteriological Control. To simplify the bacteriological aspect of this study one organism at a time was selected for treatment. A pure heat-resistant strain of *Escherichia coli*, Frank Strain No. 3636 (N. Y. Dept. of Health), was initially chosen and maintained in pure culture on peptone-glucose-beef extract slants.

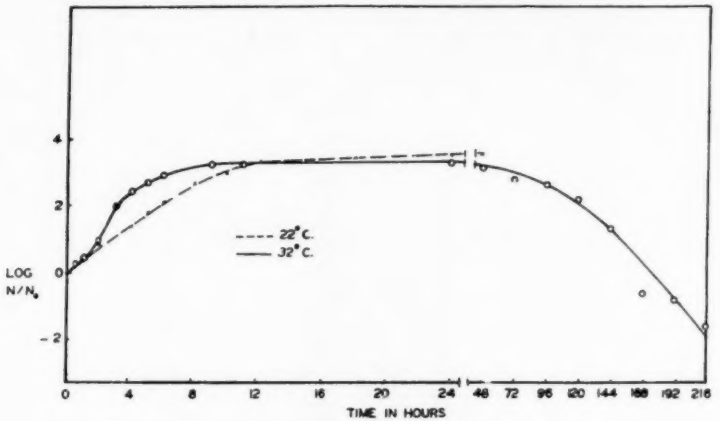

FIG. 3. Mean growth curves for *Escherichia coli*, Frank Strain No. 3636 in tryptone-glucoes-beef extract broth.

After consideration of the various methods for determining the bacterial population, the plate count was selected. A statistical analysis of the plate count showed that precision indices based upon the law of normal frequency distribution could be validly applied when the minimum plate count was 40. A confidence interval of twice the percentage standard deviation of the mean was selected. For a maximum count of 200 on each of three plates this was calculated to be ± 25%, which was considered adequate for this study. In order to assure these limits of accuracy the following techniques were necessary: 1. the use of glass beads in all dilution bottles as a precaution against clumping, 2. the use of volumes, no smaller than 1 ml., of bacterial suspension and 99 ml. of sterile distilled water for dilution, and 3. the preparation of at least three plates of tryptone-glucose agar for each of three successive dilutions differing by a factor of 10, the population being calculated from the dilution having between 40 and 200 colonies per plate after incubation at 32°C. for 48 ± 3 hours.

To determine the extent to which the physiological age and the population might be standardized by pre-radiation culturing procedure, growth curves were determined at 22°C. and at 32°C. Peptone-glucose-beef extract broth cultures were incubated for 24 hours at 32°C. A suspension of these cells in *M*/15 phosphate buffer of pH 6.81 was used to prepare broth cultures in the logarithmic phase after two hours of incubation at 32°C. Then cultures ranging in age from 30 minutes to 216 hours at 32°C. and from 30 minutes to 48 hours at 22°C. were grown from these cells. Mean growth curves at both

temperatures, drawn from the results of seven experiments at 32°C. and six at 22°C., are represented in Figure 3.

Plate-count results showed clearly that standardization of population was not accomplished by the foregoing procedure. A rapid, accurate method of determining population was therefore required. The work of Shropshire *(10)* suggested turbidity studies. A wavelength vs. transmission study of a phosphate suspension of an 8-hour culture in a Coleman Universal Spectrophotometer Model 11 for wavelengths from 400 to 650 mμ showed the greatest absorption of light at a wavelength of 400 mμ. From the logarithm of the percentage transmission vs. population curves at 400 mμ of seven phosphate-buffer dilutions (1:2 to 1:128) of each of nineteen 24-hour cultures, a mean curve was drawn. By reference to this curve, Figure 4, the population of a bacterial suspension could be read immediately with a percentage standard error of ± 8% once its percentage transmission at 400 mμ was known.

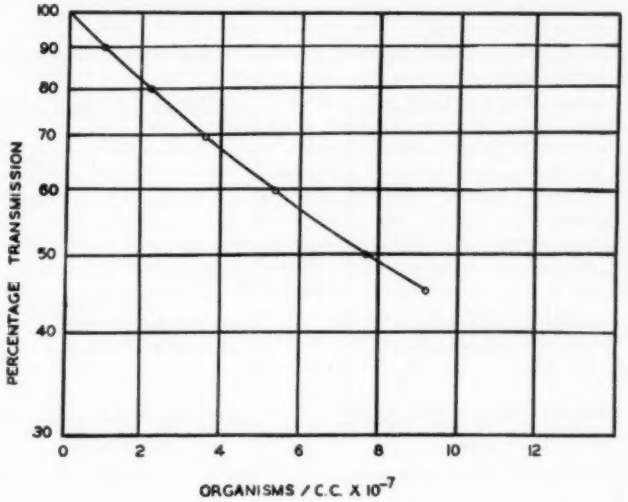

FIG. 4. Relationship between bacterial population and percentage transmission at 400 mμ for *Escherichia coli*, Frank Strain No. 3636 in *M*/15 phosphate buffer, pH 6.8.

On the basis of these studies the following culturing procedure was adopted for the standardization of the physiological age and the population prior to radiation. A selected volume of 24-hour 32°C. broth culture was spun down in an International No. 2 centrifuge at 2700 r.p.m. The cells were separated from the supernatant broth, resuspended in an equal volume of phosphate buffer, and then used to inoculate more sterile broth for 22°C. cultures of the desired age. These cells were given the additional treatment of a washing in buffer before final resuspension in buffer. A portion of the resulting suspension was diluted to allow the determination of its percentage transmission in the most accurate range and from this figure its population was read from the curve, Figure 4. For irradiation a selected volume of buffer was inoculated with a sufficient volume of this suspension to give the population desired.

Radiation Studies. To evaluate the effectiveness of the control established, radiation studies were performed on phosphate suspensions of third generation cultures (216 ± 36 min. at 22°C.) prepared as previously described and having an initial population of 1×10^8 organisms per ml. The glass radiation chamber, selected for visibility in these initial studies, was sterilized with chlorine water (200 p.p.m.) and

rinsed with sterile distilled water containing 1 ml. of 0.1 *N* thiosulfate per 200 ml. The bacterial suspension was first sampled for plate count and then transferred to the covered sterile radiation chamber. A frequency of 1000 k.p.s., a power input of approximately 500 watts, half-wavelength plates for maximum transmission, and a radiation period of 30 minutes were selected as conditions of radiation. The voltage was generally constant to within 50 volts in 1700 but the current variation was from 20-120 m.a. with an average of about 50 m.a. in 300. The volume of suspension was either 500 or 250 ml., both steel and glass plates were used, and the distance between the crystal and the chamber floor was varied from a maximum of approximately 8 cm. to a minimum of approximately 3 cm.[b] In some studies the irradiation was automatically interrupted every five minutes for sampling and temperature recording, while in others radiation was not interrupted and only the initial and final suspensions were sampled. In the former case, by continuing cooling during the sampling period it was possible to control the temperature rise somewhat so that the final temperature was not above 56°C. Under 15 minutes of continuous radiation, an the other hand, the temperature rose as

[b] Generally the oil bath and suspension were close to room temperature at the beginning of the irradiation.

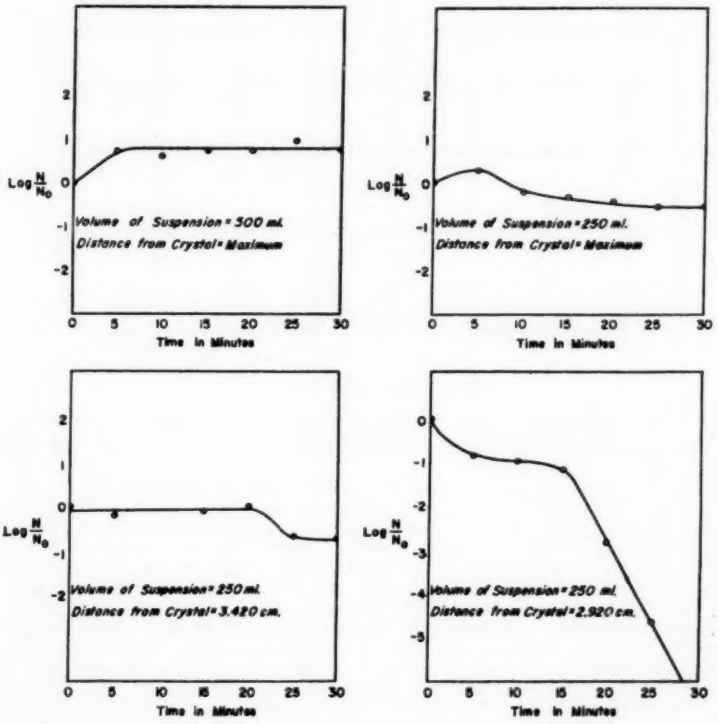
Log N/N0
Volume of Suspension = 500 ml.
Distance from Crystal = Maximum
Time in Minutes
Volume of Suspension = 250 ml.
Distance from Crystal = Maximum
Time in Minutes
Volume of Suspension = 250 ml.
Distance from Crystal = 3.420 cm.
Time in Minutes
Volume of Suspension = 250 ml.
Distance from Crystal = 2.920 cm.
Time in Minutes

FIG. 5. Effect of time of irradiation on bacterial population. *Escherichia coli*, Frank Strain No. 3636 in *M*/15 phosphate buffer, pH 6.8, 1×10^8 organisms/ml., 500 watts D. C. input.

high as 67°C. Graphs showing the rates of destruction for four experiments are presented in Figure 5.

DISCUSSION OF RESULTS

Intensity Control. While it is apparent from the results obtained that reproducibility and control of the intensity of the ultrasonic waves was not achieved, the use of half- and quarter-wave plates has made possible the elimination of any uncertainty due to the variation in the shape and thickness of the chamber floor. The measurement of distances with the cathetometer eliminated the uncertainty in these values, but the weight of the chamber was such that these distances could not be reproduced with the sample mounting used. Some consideration was given to the construction of a suitable chamber mount which would be capable of the fine and reproducible adjustments necessary to differentiate between total wave paths varying by less than a quarter of a wave-length (approximately 0.01 mm. in oil). However, the cost and time requirement of such a mount made it impractical. Therefore the control of the distance relationships, at least on the micro scale, must be considered inadequate.

The unsteadiness of the input current indicates the instability of power input. This difficulty possibly arises from the load placed on the crystal by the radiation chamber. At any rate, the contribution of power input to the ultrasonic intensity was never sufficiently stable to be reproducible.

Temperature Control. The degree of temperature control attained was not adequate for bacteriological studies. The problem of temperature control was complicated by the fact that all the heat is created in the path of the beam between the source and the surface of the liquid in the chamber. Because of the geometry of the transducer unit and the chamber, this is the most difficult region to cool. The possibility of offering interference to radiation in the chamber by possible reflection effects etc., caused a certain reluctance to placing a coil in the bacterial substrate itself, and from the experiments of Wood and Loomis *(14)* jacketing without refrigeration presented little possibility of effective cooling. Though the circulation of ice water through the bath was not considered as efficient a method of cooling as the circulation of the oil itself under optimum conditions, it seemed possibly adequate for short periods of radiation. Though the crystal has been protected from serious off-resonance vibration, the cooling achieved has not been sufficient to eliminate heat as a partial factor in destruction nor to prevent changes in the wavelength of sound in the oil, or in the height of the liquid in the chamber.

Bacteriological Control. The bacteriological control attained has been satisfactory. The culturing procedure employed controls the previous history and environmental conditions of growth of the organisms. As can be observed in Figure 3, the growth curve at 32°C. yielded a one-hour logarithmic growth phase and a generation time of 19.3 minutes which is too short for a practical standardization of physiological age. At 22°C., however, the logarithmic growth phase was lengthened to 8 hours and the generation time to 72 minutes which allows a practical working limit of ± 36 minutes in the time at which growth in the broth is interrupted and the physiological age is fixed. The determination of the initial population by measurement of the turbidity of the suspension followed by

confirmation with plate counts was designed to effect its standardization. The agreement in most cases was good. The apparent increase in population in the first curve, Figure 5, can perhaps be explained as dispersion of cells initially present, a phenomenon observed consistently by Shropshire *(10)*. The result of modifying the pre-radiation culturing procedure so as to effect complete dispersion before radiation can be seen in the other curves. Apparently the sterilization procedure and precautions against contamination during treatment were effective since only rarely were contaminants observed on the plates.

Radiation Studies. While the radiation studies serve primarily as an illustration of the degree of control obtained, some observations should be noted. Though no correlation of the rate of destruction with micro changes in distance is possible, macro changes in the distance between the crystal and the bottom of the chamber seem to have a distinct effect on the rate of destruction. When the plate was just under the surface of the oil, approximately 8 cm. above the crystal, 30 min. of radiation gave 64% destruction; at 3.420 cm., 84% destruction; at 2.920 cm., 99.99% destruction. Three runs at 3.1 cm. resulted in better than 99.99% destruction in 30 minutes in one case and in 15 minutes in two cases. These results may be due to fortuitous distance relationships which gave maximum intensity of radiation, or they may be due to the shorter distance traveled by the beam with the accompanying reduction in the amount of energy absorbed by the oil. The effect of the height of the suspension in the chamber could not be evaluated in terms of micro changes, but a decrease of 50% was responsible for an increase in the degree of destruction from practically zero to 64% when the distance between crystal and chamber was great. Very marked increases in fountain height, stability of current, rate of temperature rise, and rate of destruction of organisms were observed when the steel half-wavelength plate was substituted for the glass half-wavelength plate. Since both plates were ground to the same tolerance requirements, and since power input (as read on meters), volumes, and distance relationships were substantially the same in both cases, it is difficult to offer any explanation of this apparent increase of intensity accompanying the use of a steel plate. The effect of the final high temperatures cannot be evaluated. Pasteurization temperatures reached in two cases were of such short duration that they can hardly account for the bactericidal action obtained.

In view of the number of factors which could not be controlled and which must be controlled for quantitative interpretation of results, this study indicates the following modifications as necessary: the use of an intensity gauge, the simplification of the radiation system so that energy losses are minimized by reducing the number of interfaces crossed by the beam and so that distance relationships are fewer and more simply reproducible, temperature control within $\pm$ 1° for periods of 30 to 60 minutes in both bath and suspension, and stabilization of power input.

SUMMARY

Theoretical considerations and previous experimental work point to the necessity for control of intensity, temperature, and bacteriological factors

in quantitative studies. The present experimental efforts to achieve these conditions consist of: 1. the design and construction of a radiation chamber fitted with a series of plates which are calculated to transmit either maximum or minimum energy; 2. the measurement of all important distance relationships; 3. a glass cooling coil immersed in the oil for temperature control; 4. procedures for the standardization of physiological age, initial population, composition, and properties of the suspending medium, and methods for determining the population.

Radiation studies indicated that adequate temperature control, stabilization of input power, and reproducibility of micro-distance relationships for intensity control were not possible with the apparatus employed without major changes in the transducer assembly. In view of the fact that most uncertainties in control, barring only the direct action of heat on the suspension, are ultimately reflected in intensity, an intensity gauge is expedient for quantitative work.

Acknowledgments

The authors are indebted to Professor Arthur M. Buswell, Chief, State Water Survey; Professor Paul H. Tracy, Head, Div. of Dairy Technology, and Professor Louis B. Howard, Head, Dept. of Food Technology for administrative responsibility in arranging cooperation for this study; to Professors H. N. Hayward, W. J. Albright, and other members of the staff of the Measurement and Control Program of the Engineering Experiment Station for servicing and repair of the Ultrasonorator; to Professor William J. Fry of the Electrical Engineering Dept. for technical advice and consultation, and to Professor W. O. Nelson of the Dept. of Dairy Bacteriology for the original culture of the organism used in these studies. The interest and cooperation of Dr. P. Payson of the Crucible Steel Co. of America, Mr. Charles Weinreich and Mr. Harry Lingle of the Research Dept. of Cherry-Burrell Corp., and Dr. H. R. Lillie of the Physical Laboratory, Corning Glass Works, for their aid in the construction and design of the radiation chambers is gratefully acknowledged.

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BACTERIOLOGICAL STUDIES ON MARGARINE[a]

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A review of the literature on the microbiology of margarine showed that the major research on this important food has been done in Europe on a product considerably different chemically and produced under entirely different technical conditions from present-day American margarine. Among the more important contributions are those by Eber *(1)*, Zoffman *(14)*, Söhngen *(11)*, Jacobson *(5)*, Stokoe *(13)*, Laxa *(7)*, Horovitz-Vlasova, Morozova, Oberhard, Remennikova and Rodionova *(4)*, and Mareš *(10)*. No reference could be found to a microbiologic survey of "modern" margarine in the United States.

This investigation was carried out to gain information relative to the microbiologic status of present-day margarine. It was felt that standard plate counts at 32°C. and 35°C. incubation, coliform counts, yeast and mold counts on potato dextrose agar, microscopic mold mycelium counts, and microscopic counts of bacteria should give an overall picture of the microbiologic status of margarine.

DETERMINATION OF NUMBERS AND TYPES OF MICROORGANISMS

Samples. Fifty random samples of margarine were secured at the consumer level from local markets and from markets within a 200-mile radius of Manhattan, Kansas. An effort was made to secure samples from as many different manufacturers as possible. All samples were kept unopened in the original wrappings in a household refrigerator at about 8°C.(45°F.) until examination was made.

Tests. The standard plate count for bacteria was made according to the standard procedure used for detecting yeasts and molds in butter *(12)* except that standard tryptone-glucose-extract-agar was employed instead of potato dextrose agar. Just before pouring the plates one ml. of sterile skimmed milk was added to 100 ml. of the agar medium. Inasmuch as 32°C. and 35°C. are recognized as standard temperatures of incubation by the American Public Health Association *(1)*, it was deemed desirable to carry out incubation of the plates at these two temperatures to see if there was any material difference in the results obtained.

Coliform counts were determined by plating appropriate dilutions of the samples in Difco violet-red bile agar with incubation at 35°C. for 24 hours.

Counts of viable yeasts and molds were made by plating out appropriate dilutions of the sample in potato dextrose agar according to the standard procedure *(12)*, incubating at 25°C. for three days, and reading; then rechecking at the end of five days of incubation.

Microscopic counts of mold mycelia were made according to the Standard Methods technic using carob bean-gum solution and the Howard mold counting chamber.

Microscopic counts of bacteria were made using the Lord and Smull *(8)* modification of Fay's method *(3)*.

In all plating technics, dilutions of 1 ml., 0.1 ml., 0.01 ml., 0.001 ml., and 0.0001 ml. were employed.

Table 1 presents the results of the examinations of the 50 samples of margarine.

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[a] Contribution No. 266, Department of Bacteriology, Agricultural Experiment Station, Kansas State College, Manhattan, Kansas.

TABLE 1

Results of Examination of 50 Margarine Samples

Sample No.	Plate Count per ml. on Standard Milk Agar at		Coliform Count on Violet-Red Bile Agar	Yeast and Mold Plate Count[b]	Microscopic Count of Bacteria	Microscopic Fields Positive for Mold Mycelia, %
	35°C.	32°C.				
1	360	370	0	0	7,300,000	0
2	1,880	2,420	0	0	1,240,000	0
3	204,000	207,000	0	930Y	1,480,000	0
4	146	100	1	0	1,600,000	0
5	4	2	0	0	2,240,000	0
6	230	200	0	108,000Y	2,760,000	0
7	325,000	160,000	0	1,600Y	2,160,000	0
8	2,150,000	1,920,000	0	1M	4,000,000	0
9	4	2	0	0	2,480,000	0
10	4	7	0	0	3,160,000	0
11	600	250	0	0	1,760,000	0
12	1,110	1,400	0	1,220Y	10,000,000	4
13	210	190	0	0	600,000	0
14	5,300,000	6,200,000	0	5,000,000M	1,100,000	0
15	30	28	0	0	240,000	12
16	320,000	350,000	0	2M	1,500,000	0
17	100	10	0	0	3,000,000	2
18	8	12	0	30M	400,000	0
19	4,470,000	4,500,000	0	5M, 5Y	1,200,000	0
20	1,350,000	1,530,000	0	3,800Y	680,000	6
21	1,990	1,620	0	0	400,000	4
22	380	160	0	0	960,000	0
23	30	28	0	1M, 5Y	2,100,000	0
24	1	10	0	1Y	7,700,000	0
25	Large[c]	39	0	7M, 1Y	1,000,000	0
26	1	1	0	1M	1,040,000	2
27	43,000	49,000	0	0	1,080,000	2
28	1	1	0	9Y	380,000	0
29	1	11	0	2M	480,000	4
30	72	320	0	0	2,300,000	2
31	8,000	8,400	0	4Y	2,240,000	4
32	1	1	0	0	720,000	2
33	40	39	0	0	2,080,000	12
34	5,400	6,000	0	12Y	880,000	0
35	1	4	0	0	4,040,000	0
36	40	22	0	0	480,000	0
37	1	1	0	0	880,000	8
38	460	280	0	0	2,900,000	2
39	10,200	420	6	10M, 25Y	1,280,000	0
40	30,000	37,000	0	0	1,900,000	0
41	100	900	0	0	600,000	4
42	690	820	0	1M	3,300,000	2
43	8,900	880	0	1Y	3,040,000	2
44	10	310	0	0	1,900,000	4
45	125,000	289,000	7	0	7,360,000	0
46	96	38	0	3M	11,200,000	4
47	60	60	0	14M, 1Y	5,800,000	2
48	2,490,000	3,080,000	40	161,000M	8,720,000	4
49	1,400	1,280	0	1M	1,040,000	0
50	510	410	0	1M, 1Y	1,440,000	0
Arithmetical mean	344,000	366,980			2,560,000	
Logarithmic mean	531	523			1,700,000	

[b] M indicates mold colonies and Y indicates yeast colonies per ml. [c] Too numerous to count in that dilution.

Attention is called to the wide variation in the counts of the different samples. In the column headed "Plate count per ml. on standard milk agar 35°C.," Sample 24 showed only one bacterium per gram and Sample 14 showed 5,300,000 bacteria per gram. Twenty-one samples gave counts of 100 or fewer. This represents almost 50% of the samples.

Attention is next called to the column headed "Plate count per ml. on standard milk agar 32°C." In general these counts parallel those obtained at 35°C. In each instance, except sample number 25, the results are of the same magnitude. When either the arithmetical or logarithmic means are compared, one finds that there is little if any significant difference between results obtained at 32°C. or at 35°C. incubation.

The violet-red bile agar plate counts for coliform bacilli showed only four positive samples out of the 50 samples examined.

The potato dextrose agar counts for yeasts and molds produced 27 negative samples and 17 samples with from one to only a few colonies on the 1 ml. plate.

The microscopic counts of bacteria varied from 240,000 to 11,200,000, with a logarithmic mean of 1,700,000 per ml. of melted margarine.

Twenty-nine of the 50 samples were negative by the microscopic mold mycelium determination. The highest value was 12% positive fields on Samples 15 and 23.

DETERMINATION OF SPECIES OF BACTERIA IN MARGARINE

During the course of the study, colonies appearing on the standard milk agar plates at 32°C. and 35°C. were picked at random and transferred onto nutrient agar slants. An attempt was made to select three or four colonies typical of the major types appearing on the plates of each sample. These colonies were then identified taxonomically, using the procedures and media cited in the Manual of Methods for Pure Culture Study of Bacteria. From the 50 margarine samples, 180 aerobic cultures were isolated. Table 2 shows the major species isolated and the percent of samples yielding each particular species.

TABLE 2

Prevalence of Species of Bacteria Isolated from Margarine

Species	Isolated from Percent of Samples
Bacillus alvei	18
Bacillus cereus	38
Bacillus laterosporus	10
Bacillus megatherium	34
Bacillus pumilis	18
Bacillus subtilis	10
Microbacterium flavum	10
Micrococcus aurantiacus	22
Micrococcus candidus	22
Genus Bacillus	72
Genus Micrococcus	56

Of the samples investigated, 72% contained members of the genus *Bacillus*, and 56% of the samples contained members of the genus *Micrococcus*. The following species were isolated from less than 10% of the samples: *Achromobacter stationis, Alkaligenes marshalli, Alkaligenes metalkaligenes, Bacillus brevis, Bacillus circulans, Bacillus coagulans, Bacillus firmus, Bacterium linens, Corynebacterium helvolum, Escherichia coli* var. *communior, Escherichia coli* var. *neapolitana, Flavobacterium aquatile, Flavobacterium breve, Flavobacterium dormitator, Flavobacterium lutescens, Flavobacterium proteus, Flavobacterium sp., Micrococcus caseolyticus, Micrococcus citreus, Micrococcus epidermidus, Micrococcus flavus, Micrococcus freudenreichii, Micrococcus luteus, Micrococcus pyogenes* var. *albus, Micrococcus varians, Nocardia paraffinae, Pseudomonas fragi, Pseudomonas nebulosa, Sarcina flava, Streptococcus mitis, Streptomyces olivaceous.* Altogether, 40 different species were isolated.

LIPOLYTIC AND CASEOLYTIC CHARACTERISTICS OF MARGARINE BACTERIA

Söhngen *(11)* reported that fat-splitting microorganisms could affect the flavor of margarine. With such a wide variety of organisms present in margarine, it was deemed advisable to see what percentage of the isolated strains could attack fats. Each culture was streaked on a tallow agar plate and incubated for 5 days at 35°C. The plates were read for hydrolysis of the tallow. Table 3 presents the results of the tests on lipolytic activity. It may be seen that only 13.33% of the cultures were lipolytic.

TABLE 3

Results of Tests on Lipolytic and Caseolytic Activity of 180 Organisms Isolated from Margarine

Property	Number of Cultures	Found in Percent of Cultures
Lipolytic Activity		
Actively lipolytic	21	11.66
Weakly lipolytic	3	1.66
Non-lipolytic	156	86.66
Caseolytic Activity		
Caseolytic	99	55.0
Non-caseolytic	81	45.0

Kerr *(6)* substantiated Söhngen's *(11)* claim that rancidity changes were not all the result of lipolysis but might come also from bacterial decomposition of protein residues in the margarine. Because a certain amount of curd is left in the finished margarine, it was thought a study of the number of caseolytic organisms might give some idea as to the potentiality of bitterness or putrid odor defects in margarine arising from proteolytic bacteria. The 180 cultures were streaked onto a medium composed of 50% skimmed milk and 1% agar-agar. A zone of digestion of the casein of the milk-agar medium indicated caseolytic activity. Table 3 presents the results of this property. Slightly over half the strains were caseolytic.

DISCUSSION

The general low plate count values may be explained in part on the general practice of adding 0.1% sodium benzoate to the finished margarine. However, variations in the counts of from a few to a few millions of bacteria per ml., suggest that sanitary practices during manufacture and storage temperatures may have influenced the microbic populations significantly. Horovitz-Vlasova, *et al. (4)* have pointed out the need for cleanliness in raw materials, surroundings, and wrapping to obtain good keeping quality.

The rather uniformly low coliform count indicates a product probably free of fecal or manurial pollution.

The viable yeasts and molds present may have been derived from the churning milk or may have come from improper handling in the factory. It is impossible to tell from which source they came. Only three samples showed abnormally high yeast or mold counts. *Penicillium* and *Aspergillus* species predominated in the mold flora. The yeasts were not identified taxonomically. Dust contamination during manipulation could account easily for the occasional mold or yeast colony on a plate.

Interpretation of the high microscopic count of bacteria should be made with care. One must keep in mind that a cultured milk containing millions of bacteria per ml. is used in the churning of the margarine. Obviously many of the bacteria contained in the cultured milk will be present in the finished product. If they are killed by the sodium benzoate, they will fail to grow upon culturing, but the dead cells will be present. Normally, the microscopic count of a processed food (which tests as "satisfactory" upon culturing) can be used to detect a product made from poor grade raw materials. This interpretation cannot be made here any more than it could be made for butter or cultured buttermilk. The presence of "foreign" species of bacteria would indicate a possible "unsatisfactory" condition during manufacture.

It is possible that mold mycelium determinations may be of special significance in margarine examinations. The mold count was consistently low in these 50 samples. Even 12% positive fields is insignificant when compared with values obtained from the examination of other processed food products. If it be assumed that these 50 samples were of good quality, then the occurrence of a high mold count might be interpreted as indicating poor quality.

With the possible exception of the genus *Micrococcus*, the bacterial flora of margarine appears to be composed of types usually considered as "harmless." However, one must not overlook the possibility of contamination of the product by use of contaminated churning milk, improperly handled oils, or faulty hygiene of factory workers.

CONCLUSIONS

From the results of the examination of 50 random samples collected at the consumer level on local markets, present-day margarine appears to have a microbial population similar in magnitude and variety to that of many other processed foods; and in some respects its bacteriological status may be even superior to that of many other foods.

Of the 50 samples of margarine examined, 42% gave plate counts of 100 bacteria or less per ml. There was little significant difference in results obtained by incubating plates at 32°C. and 35°C.

Coliform organisms were isolated from only 8% of the samples and then only in very small numbers.

Fifty-four percent of the samples were negative for viable yeasts and molds upon culturing, and 58% were negative microscopically for mold mycelia, and the highest value obtained was 12% positive fields.

Microscopic counts of bacteria varied from 240,000 to 11,200,000 per ml.; however, the microscopic count probably cannot be considered as a valid test for judging sanitary quality of margarine.

Forty different species of bacteria were isolated. Fifty-five percent of the cultures isolated were caseolytic and 13.33% were lipolytic. Members of the genus *Bacillus* were found in 72% of the samples, and the genus *Micrococcus* was isolated from 56% of the samples.

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THE MECHANISM OF MEAT-PIGMENT OXIDATION. THE EFFECT OF SOLUTES ON THE HEMOGLOBIN-OXYGEN EQUILIBRIUM

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The benefits derived from the use of sodium chloride in fresh meat are well known. Less well known and only incompletely understood is the fact that salt increases the rate of oxidation of the red meat pigments, oxyhemoglobin and oxymyoglobin, and/or hemoglobin and myoglobin, to the brown methemoglobin and metmyoglobin, respectively. Understanding the mechanism by which salt causes this effect constitutes the objective of this study.

Among the first to have recorded this effect of salt were Stiles and Foster *(37)* and Brooks *(7, 8, 12)*. The former workers showed that alkalinization of brine containing beef significantly reduced this salt effect, and hence concluded that such an effect by salt was due simply to the lowering of pH. Brooks essentially confirmed this conclusion. He calculated *(9, 13)*, from experiments involving 4 and 10% salt in phosphate-buffered blood solutions, that approximately 20 and 62%, respectively, of the increased oxidation rate was not due to alterations in pH.

Finally, in this laboratory, meat, the surfaces of which were dusted with salt (C. P.), was stored under conditions excluding bacterial interference, and such surfaces observed. Those surfaces containing salt were found to discolor to the brown "met-pigment" color more rapidly than those not containing salt. Further, salt was found to favor this discoloration in fresh aqueous beef extracts.

It appeared, then, that sodium chloride exerted its undesirable effect upon meat color through, possibly, several pathways. These pathways, however, might not be unrelated and might be operating through the same mechanism considered to operate under another better-known condition causing accelerated oxidation; namely, low oxygen pressures. Such a possibility was explored.

If a section be made of a well-packed loaf of ground fresh beef tissue after its storage for a few days above freezing temperatures, a narrow brown ring, following closely the periphery of the loaf, can be observed. The principal color components of this brown ring are the brown-colored oxidized pigments, methemoglobin and metmyoglobin. Inward from this ring can be seen the typical red-purple color characteristic of hemoglobin and myoglobin, whereas between it and the surface can be noted a narrow bright red band containing the bright red pigments, oxyhemoglobin and oxymyoglobin. Because reducing enzymes within the tissue produce an oxygen concentration gradient extending from the surface to the interior, as shown by Warburg *(40)* and Hill *(24)*, the conclusion follows that a lowered oxygen pressure accelerated the oxidation of the pigments in the brown ring.

Credit for the first clue that ease of oxidation of the red oxyhemoglobin and oxymyoglobin to their corresponding brown "met" derivatives might increase under environments of low oxygen pressures should probably be given to Conant and Fieser *(16)*. They found that whereas hemoglobin, in the absence of any gas with which to combine, could be completely oxidized by one equivalent of ferricyanide, 13.5 of such equivalents were required at oxygen pressures near one atmosphere.

Further work by Neill and Hastings *(32)*, Brooks *(5, 6, 10, 11, 14)*, and Legge *(29)* added strength to this belief. They observed that the rate of oxidation of hemoglobin by oxygen reached a maximum at the low oxygen pressures of 4 mm. and 20 mm. Hg. at temperatures of 0°C.(32°F.) and 30°C.(86°F.), respectively. This evidence permitted Neill and Hastings, and Brooks to suggest that the brown methemoglobin was formed by the oxidation of hemoglobin rather than of oxyhemoglobin. Criticism of this mechanism by Lemberg, Legge, and Lockwood *(30)* and Legge *(29)* suggested that, of the various intermediates [Hb_4, $Hb_4(O_2)$, $Hb_4(O_2)_2$, $Hb_4(O_2)_3$, and $Hb_4(O_2)_4$] which exist under the conditions producing the maximum rate of hemoglobin oxidation, the concentration of $Hb_4(O_2)_2$ exceeded that of all others. They claimed that the oxidation of this half-oxygenated component was the rate-determining step. Some older work of Conant and McGrew *(17)*, however, considers unlikely the formation of the hemoglobin-oxygen intermediates other than Hb_4 and $Hb_4(O_2)_4$.

Of significance for this study was the fact that whichever of the two cited mechanisms operated at low oxygen pressures, increase of the concentration of the oxygen-unsaturated components, Hb_4 or $Hb_4(O_2)_2$, led to an increase in the rate of methemoglobin formation. Not to be ignored was the possibility that sodium chloride caused this same discoloration by also increasing the concentration of these components. If salt did act thus, oxygen would be evolved when salt is added to oxyhemoglobin at atmospheric oxygen pressures. Hence, this hypothesis was tested by the measurement of gas evolution resulting from the addition of sodium chloride and other solutes to defibrinated laked blood at atmospheric oxygen pressures.

Measurements of the effect of sodium chloride on the hemoglobin-oxygen equilibrium have been recorded previously, but they have been somewhat inconsistent. Barcroft and Camis *(3)* found that sodium chloride (0.7%) increased the affinity of hemoglobin for oxygen within the oxygen pressure range of 5-90 mm. Hg., but observed no effect on the equilibrium at higher oxygen pressures. The investigations of Kono *(27)*, Guzman-Barron, Munch, and Sidwell *(22)*, and Sidwell, Munch, Guzman-Barron, and Hogness *(35)* showed that at low oxygen pressures (5-16 mm. Hg.) sodium chloride additions up to 1.4% led to a lesser affinity of hemoglobin for oxygen. In experiments involving a wider oxygen pressure range and in which were employed sodium chloride concentrations up to 12%, Green and Talbott *(21)* also found similar dissociating effects.

None of these investigations provided, however, the information sought in this study since sufficiently high concentrations of sodium chloride were not added to the hemoglobin-oxygen equilibrium at atmospheric oxygen

pressures. Further, buffers were added to the hemoglobin solutions in most of these prior investigations to prevent alterations in pH by solutes. In this work, such use of buffers was avoided because added buffers were absent in salt-induced oxyhemoglobin oxidations and because information about the production of oxygen-unsaturated hemoglobin components at atmospheric oxygen pressures, whether by direct solute effect or pH change, was valuable for the purposes of this investigation. It should be noted that the dissociation of oxyhemoglobin at atmospheric oxygen pressures is relatively insensitive to minor alterations in pH [Adair *(1)*, Adolph and Ferry *(2)*, Ferry and Green *(19)*, Forbes and Roughton *(20)*, Hill *(23)*, Kuznetsov *(28)*, Rona and Ylppo *(34)*].

EXPERIMENTAL

A constant pressure micro-respirometer of the type devised and described by Dixon *(18)*, except for a few modifications, was built for this study. The micro-flasks, possessing comparable volumes, were bifurcated at the base to the extent that within each flask, two 1-ml. portions of solutions could be separately contained and yet yield to complete mixing when agitated. Those flasks which served as reaction vessels were provided with a single glass-stoppered side-arm so situated so as to provide for easy additions of solutions into both chambers by means of an extended pipette tip. The capillary tubing, within which the volume changes were measured by adjustment of a mercury reservoir, was carefully selected and possessed a uniform bore diameter of 1.0 mm.[a] The mercury level changes in this tubing, which were related to the volume of gas formed or absorbed, were measured on a background of millimeter graph paper.

For the purpose of this study, a stable blood solution as free of methemoglobin as possible was necessary. Such was obtained by distributing 5-ml. portions of defibrinated, laked, bovine blood solution[b] among many cork-stoppered test tubes which were then stored at −50°C.(−58°F.) in a solid carbon dioxide chamber. Periodic oxidation[c] of these samples by excess ferricyanide (80 and 120 x theory) by means of the respirometer produced insignificant variations in the volumes of oxygen evolved[d] when such samples were analyzed over a one-month period. Hence, the extent of oxidation of the stored frozen blood during the period of this study was considered unimportant.

Since the bicarbonate-carbon dioxide system in the blood could also be affected by solutes, a blood "blank" was sought which permitted isolation of the solute effect on the oxyhemoglobin equilibrium. The "blank" chosen consisted of stored blood samples to which the minimum volume of concentrated ferricyanide was added which caused complete oxidation so that, in effect, oxyhemoglobin had been replaced by methemoglobin. Inasmuch as solutes were to be added to this ferricyanide-containing blood "blank" on the one hand, and to the blood solution not containing ferricyanide on the other hand, it became necessary to prove that any effects due to the ferricyanide, itself, could be ignored. Respirometric experiments in which saturated salt and

[a] A very exact measurement of this capillary diameter was unnecessary since comparisons between calculated volumes, all of which contained the same diameter term, constituted the major objective.

[b] Freshly-drawn bovine blood was defibrinated by whipping with stick applicators and, after removal of most of the separated fibrin, was passed twice through cheese cloth. The resulting erythrocyte suspension was then laked by the alternation of freezing and thawing.

[c] The conditions and procedures which were employed in these determinations are described in greater detail in connection with the experiments involving the measurement of solute effects.

[d] The mean volume-percentages of oxygen evolved from the oxyhemoglobin solution after oxidation by the solutions containing the lower and higher ferricyanide concentrations were 16.8 and 17.4, respectively, values found not to be significantly different statistically. These values were based upon the volume of undiluted blood solution.

other solute solutions were added to sera in which ferricyanide was either present or absent offered such proof.

The procedure employed for determining the salt effect, and which was followed for other solutes as well, was as follows:

A test tube (12 mm. x 115 mm. ligature tube) of the frozen laked blood was, while still in the dry-ice box, unstoppered and quickly placed within another test tube (15 mm. x 125 mm.) which, in turn, was then stoppered. This double test tube was permitted to remain at room temperature for 20 minutes during which period the rise of the temperature of the contents to that of the room was hastened by frequent warming with the hand. Into a ligature tube was pipetted 0.0625 ml. distilled water and 1.25 ml. of the blood solution after which the tube was so suspended in an extension from a horizontal shaker that, during the shaking[e] of the tube in a water bath at 23.00° ± 0.01°C.(74°F.) for 40 min., part of the blood mixture distributed itself as a thin film over the inner wall. After such shaking had ceased, a timed one-half hour period was allowed for pipetting[f] 1.00 ml. of such shaken blood solution and 1.00 ml. of 2 *M* sodium chloride solution[g] into each of the two chambers of the mixing flask by way of the side-arm.

If, during this timed half-hour period, replacement of the side-arm plug followed by closure of the two stop-cocks did not lead to significant pressure variation[h], then, just prior to this period's end, the two stop-cocks were opened and the mercury level adjusted to the desired reading. When the period terminated, the stop-cocks were closed and the respirometer shaken vigorously by hand in a manner which permitted the flasks to retain essentially their original depth in the water bath. The barometric pressure and temperature of air near the mercury column were recorded[i] and a timer set to permit the solute to exert its effect for one hour. The respirometer was shaken frequently during this time and following each shaking a coarse adjustment of the mercury level was made, if required, to retain within the mixing chamber the original barometric pressure[j].

Finally, after this 1-hour period had elapsed, the mercury level was so adjusted that no difference in the paraffin levels in the two arms of the manometer existed. Under these conditions, the pressure of the gas formed or absorbed was that of the prior recorded barometric pressure. This final adjusted mercury level and the air temperature were then recorded. The volume of the dry gas produced or absorbed under S.T.P. conditions was calculated with the aid of the gas laws in the usual manner[k].

RESULTS AND DISCUSSION

Determination of the volume effects produced by the 2 *M* sodium chloride solution on the "blank" containing methemoglobin was obtained by

[e] The shaker oscillated horizontally at a rate of approximately 250 cycles per minute. The shaking time of 40 minutes was controlled by an automatic clock timer.

[f] The pipettes employed were of the 1 ml. holding type, the ends of which had been drawn out so that samples involved in a determination could be both accurately and easily dispensed into the separate flask chambers.

[g] All solute solutions employed in this study were contained in vessels immersed in the water bath at 23.00° ± 0.01°C.(74°F.).

[h] With the aid of fixed crossbars, the respirometer, after shakings, was always restored to the same position. This was important since the pairs of flasks employed were not identical in volume, and the temperatures of the room and water bath could not be maintained at equal values.

[i] Since the volumes above each arm of the paraffin-containing manometer were considerably different, the air temperature was purposely maintained within a few tenths of one degree C. during the 1-hour shaking period.

[j] Most of the volume change produced after mixing occurred within the first 15 minutes of the 1 hour mixing period.

[k] For the purposes here, the vapor pressure of the water in the blood-solute mixtures was considered equal to that of pure water at the same temperature. The error introduced by this assumption is unimportant since water at 20°C.(68°F.) has its vapor pressure reduced by only 1.4 mm. when one-half saturated with sodium chloride.

the substitution in the foregoing experiment of 0.0625 ml. potassium ferricyanide solution[1] for the equivalent volume of distilled water. The effects of both 2 *M* and approximately 5.4 *M* (saturated) sodium chloride solutions upon the oxidized and unoxidized blood solutions were then observed and calculated. The mean effects of the 2 *M* salt solution on the oxidized and unoxidized blood solutions were 0.69 (A) and—1.21 (B) mm.^{3}, respectively. The mean effects of the 5.4 *M* salt solution on these two blood solutions were —2.54 (A) and —3.68 (B) mm.^{3}, respectively. The differences (B-A) in both instances, representing oxygen evolved (+) or absorbed (—), proved to be statistically insignificant at the 5% level. For comparison, the mean volume of oxygen liberated from the blood solution by excess ferricyanide (80 x and 120 x theory) was 170.4 mm.^{3} The best estimate of σ for this value was calculated to be 3.41 mm.^{3}

It appeared, then, that at atmospheric oxygen pressures, even a saturated solution of sodium chloride produced no significant dissociation or association effects upon the oxyhemoglobin equilibrium. It is concluded, therefore, that the accelerating effects of salt and of low oxygen pressure on the oxidation rate of oxyhemoglobin probably did not share a common mechanism. Or, if a common mechanism did exist, it was as yet unknown.

Inasmuch as oxyhemoglobin consists largely of protein, respirometric experiments, identical to those previously described but in which were employed solutions of strong denaturing agents, were performed. Both oxidized and unoxidized blood solutions were treated with saturated urea and 2 *M* guanidine solutions and the volume effects calculated. The mean effects of the 10.2 *M* urea solution on the oxidized and unoxidized blood solutions were 6.21 and —0.35 mm.^{3}, respectively. The mean effects of the 2 *M* guanidine hydrochloride solution on these two blood solutions were 4.63 and 1.74 mm.^{3}, respectively. Whereas the urea solution did cause effects in these two blood solutions which significantly differed statistically, the guanidine hydrochloride solution produced effects which did not so differ.

The action of 8 *M* guanidine hydrochloride was also sought. Its inability, however, to satisfy the preliminary requirement that no significant difference exists between its effect on sera in which ferricyanide was either present or absent, precluded its use.

Prior investigations of the effect of denaturing agents upon the oxygen-hemoglobin equilibrium have been reported. All added these agents over those ranges of low oxygen pressure where the rate of change of "percent oxygenation" with respect to "oxygen pressure" was greatest but all did not employ similar concentrations. Poulton and Ryffel *(33)*, Momose *(31)*, and Adolph and Ferry *(2)*, employing urea in concentrations as high as 1 *M*, observed no effect on the equilibrium whereas Taylor and Hastings *(38)*, and Boeri and Vescia *(4)*, studying hemoglobin solutions containing urea of concentrations 4 and 6.66 *M*, respectively, found that combination of hemoglobin with oxygen increased.

In this study, saturated urea solutions appeared to increase the affinity of hemoglobin for oxygen. This oxygenation of an additional few percent

[1] This solution contained 173 g. per 500 ml. solution.

of hemoglobin appeared to occur in spite of the fact that, at atmospheric oxygen pressures, the degree of oxygenation of hemoglobin is known to be relatively insensitive to variations in oxygen pressure.

In explanation of this urea effect, the suggestion has been offered by Taylor and Hastings *(38)* and Boeri and Vescia *(4)* that elevated urea concentrations dissociate hemoglobin into half-molecules *(15, 36, 41)*. Because these half-molecules were comparable to myoglobin in molecular weight, it was possible that they also possessed that muscle pigment's superior attraction for oxygen *(25, 26, 39)*. In any event, urea, a potent protein denaturing agent, certainly did not appear to effect the liberation of oxygen from the heme-protein, oxyhemoglobin.

The investigation of the mechanism by which nicotinic acid and its esters transform the interior color of meat from a red-purple to a bright red (U. S. Patent, No. 2,491,646), also lent itself to manometric aid. Did these compounds react with oxyhemoglobin (and oxymyoglobin) to form bright red complexes and oxygen? Or was such improved color due merely to the simple combination of these compounds with hemoglobin (and myoglobin)? Accordingly, determinations of the effects of both nicotinic acid and its methyl ester upon the oxyhemoglobin equilibrium were performed.

Measurements showed that the pH of the nicotinic acid-blood solutions employed was 2.5 pH units lower than that of the blood solution alone. To evaluate the effect of the acidity of nicotinic acid upon the oxyhemoglobin equilibrium, parallel respirometric experiments were performed employing propionic acid. This acid was chosen because it and nicotinic acid possessed identical acid ionization constants and because no evidence existed that it reacted with oxyhemoglobin. The mean effects of the 0.1 *M* nicotinic acid solution on the oxidized and unoxidized blood solutions were 47.4 and 88.3 mm.[3], respectively. The mean effects of the 0.1 *M* propionic acid solution on these two blood solutions were 48.2 and 82.6 mm.[3], respectively. The mean effects of the 2 *M* methyl nicotinate solution on the oxidized and unoxidized blood solutions were 11.7 and 73.8 mm.[3], respectively. The two acids were found to produce statistically identical effects both upon the oxidized and the unoxidized blood solutions[m]. Hence it was concluded that the dissociating effect [n] of nicotinic acid on the aforementioned equilibrium could be entirely attributed to its acid character, rather than to the formation of a nicotinic acid hemoglobin complex which was more stable than oxyhemoglobin. This conclusion in no way implied that nicotinic acid could not combine directly with hemoglobin in the absence of oxygen to form such a nicotinic acid-hemoglobin complex.

Methyl nicotinate, however, possessing no complicating acid character, did appear to cause approximately 35% of the total bound oxygen to become liberated, the difference between its effect on the oxidized and unoxidized blood solution being statistically highly significant. Inasmuch

[m] The estimated mol ratio of nicotinic acid to oxyhemoglobin in those experiments involving this acid was approximately 5.

[n] The difference between the effects of the nicotinic acid on the two blood solutions was found to be statistically significant.

as such a large effect on the oxyhemoglobin equilibrium would be unlikely without reaction, the tentative assumption is made that methyl nicotinate, under the conditions described, partly displaces oxygen from oxyhemoglobin with formation of some hemoglobin-methyl nicotinate complex. It should be noted that the concentration of methyl nicotinate considerably exceeded that of the relatively water-insoluble nicotinic acid.

SUMMARY

An attempt was made to relate two conditions under which the accelerated oxidation of hemoglobin occurred—that in which the oxygen pressure was reduced and that in which sodium chloride was present. The fact that saturated sodium chloride solutions produced no significant alteration of the oxygen-hemoglobin equilibrium at atmospheric oxygen pressures suggested that these two conditions caused this oxidative effect either through independent mechanisms or, if not independent, through a mechanism as yet unknown. A concentrated solution (10.2 *M*) of the strong protein denaturing agent, urea, was found to cause a slightly increased oxygenation of hemoglobin at atmospheric oxygen pressures. Further information was sought on the mechanism by which nicotinic acid and its lower alkyl esters transform the interior color of fresh meat from a red-purple to a bright red.

The acidity of nicotinic acid appeared to be the only factor causing dissociation of oxyhemoglobin by nicotinic acid. Other than this effect of its acidity, nicotinic acid did not appear to react with oxyhemoglobin. In contrast, methyl nicotinate, having no complicating acid property, did appear to react with oxyhemoglobin.

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STUDIES ON THE CHOLINE CONTENT OF SOME COMMON INDIAN PULSES AND CEREALS BOTH BEFORE AND DURING THE COURSE OF GERMINATION

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In our studies on the vitamin content of pulses and cereals commonly grown in India, both before and during the course of germination, we have observed that, owing to their increased vitamin content, germinated pulses are nutritionally superior to the ungerminated pulses. Nandi and Banerjee *(9, 10, 11)* found that the germinated pulses contained increased amounts of ascorbic acid (vitamin C), nicotinic acid, and riboflavin. That the germinated pulses and cereals possess an increased amount of thiamine was shown by Chattopadhyay, Nandi, and Banerjee *(3)*, while Chattopadhyay and Banerjee *(2)* showed an increased amount of carotene to be present in the germinated pulses. Choline is of nutritional significance since Best, Channon, and Ridout *(1)* showed that it possesses lipotropic activity and, according to du Vigneaud, Chandler, Moyer, and Keppel *(5)*, choline takes part in the transmethylation reaction. It was of interest, therefore, to establish the choline content of Indian pulses as well as the effect of germination on their choline content.

EXPERIMENTAL

Germination of the Seedlings. Five g. of healthy dry seeds of pulses were weighed and kept in clean petri dishes. Sufficient distilled water for germination was added daily to the petri dishes for four days. The seeds germinated well and the seedlings were healthy. The petri dishes were kept away from direct sunlight. In case of the cereals, 5 g. of healthy dry seeds were weighed and kept in petri dishes soaked with sufficient distilled water for one day. From the second day they were embedded in earth soaked with water and the petri dishes were kept away from the direct sunlight.

Extraction and Estimation of Choline. The germinated seeds were dried in an electric oven at 60-70°C.(140-158°F.) for three hours, crushed to powder with pure sand, and extracted with methyl alcohol for twenty-four hours. Choline in the extract was estimated according to the method of Glick *(7)*. During the estimation of choline in the germinated seeds it was seen that oxidized pigments interfered with the precipitation of choline reineckate. To prevent this, the methyl alcohol extract, after evaporation of most of the alcohol, was vigorously boiled with a saturated solution of barium hydroxide for two hours. This, after neutralization with acetic acid, was twice filtered through a sintered glass filter tube under suction. The filtrate, which was more or less free from darker pigments and gave a pink color, was then treated with reineckate solution as usual. The average results of the analysis of six samples in each case are given in Table 1.

In order to verify the accuracy of the method used, known amounts of choline were added to 5 g. of crushed pulses *(Lens esculenta)* and the choline was then extracted with methyl alcohol. Choline in the extract was estimated as described above and good recovery of the added choline was obtained. The results are given in Table 2.

RESULTS AND DISCUSSION

In view of the importance of pulses in the Indian dietary it was considered desirable to obtain as adequate information as possible concerning

TABLE 1

Effect of Germination on the Choline Content of Indian Pulses and Cereals

Local name	Botanical name	Choline contents (mg. per 100 g.)		
		Ungerminated	After germination for	
			2 days	4 days
Musuri	*Lens esculenta*	223±3.5	245±1.6	268±2.5
Mung	*Phaseolus radiatus*	205±2.5	230±2.2	260±2.5
Krishna mung	*Phaseolus radiatus*	213±2.3	232±2.0	261±3.1
Chhola	*Cicer arietinum*	245±4.0	262±3.3	282±2.1
Kalai	*Phaseolus mungo*	99±1.5	118±1.1	125±1.3
Arhar	*Cajanus indicus*	201±2.2	224±1.1	239±2.9
Golapi chhola	*Cicer arietinum*	215±1.1	230±1.5	250±2.2
Hara chhola	*Cicer arietinum*	235±1.8	260±1.8	281±2.6
Mashkalai	*Phaseolus roxburgi*	101±2.2	108±1.6	116±2.0
Black eyed pea	*Pisum sativum*	197±1.8	212±2.5	236±2.8
Pyramotor	*Pisum sativum*	201±1.5	220±1.6	233±1.1
Rice	*Oriza sativa*	70±1.8	80±1.5	83±1.2
Wheat	*Triticum vulgare*	70±1.8	85±1.0	90±0.8
Maize	*Zea mays*	0	0	0

TABLE 2

Recovery of Choline Added to 5 g. of Pulse (Lens esculenta)

Sample No.	Choline added	Choline estimated	Choline recovered
	mg.	*mg.*	%
1	0	11.15	.....
2	1.95	13.05	99.6
3	3.89	14.65	97.4
4	0.39	11.52	99.9
5	0.78	11.84	99.2
6	1.17	12.29	99.7

the vitamin content of these seeds and also to study the effect of germination on the same. Previous studies have shown that germinated pulses are also good sources of nicotinic acid, ascorbic acid, thiamine, riboflavin, and carotene *(2, 3, 9, 10, 11)*. The data presented in this paper clearly show that pulses are also very rich sources of choline. However, cereals are comparatively poorer in their choline content. The choline content of pulses increased during the course of germination. In cereals, also, there was a slight increase in choline content during germination. These increased choline values during germination are statistically significant. In case of corn, however, there was no choline either before or during the course of germination. This result does not agree with the observations of Engel *(6)* and Horowitz and Beadle *(8)*. This might be due to the different strains of corn grown in India. Of the pulses, *Lens esculenta*, different varieties of *Cicer arietinum*, and *Phaseolus radiatus* showed comparatively higher increases in choline content during germination. The increase was generally marked from the second day of germination. *Phaseolus mungo* and *Phaseolus roxburgi* contain smaller amounts of choline in comparison with other pulses.

SUMMARY

The choline content of eleven varieties of pulses and three varieties of cereals were estimated. Pulses are a very rich source of choline whereas cereals are poorer in their choline content. During germination the choline content of both the pulses and cereals increased significantly.

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TAINT IN SOUTHERN COUNTRY-STYLE HAMS

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Southern country-style ham is a cured product with a distinctive flavor. Like many types of cheese, it may be stored at atmospheric temperature after cure with the continued development of intensity of flavor. During the curing period, however, it is subject to several types of spoilage, chief of which is taint or souring, a proteolytic rather than a fermentative degradation brought about by bacterial action. It is impossible to state exactly the amount of meat affected in this manner, but it has been estimated unofficially that in one neighboring state alone the major percentage of approximately 10 million pounds of meat is tainted annually.[b]

Much work has been done on cured hams during the past 25 years [see, for example, the reports of Bate-Smith and co-workers in the Reports of the Food Investigating Board, as reviewed by Ingram *(8)*]. Yet, there is little information regarding the causative organisms of taint, particularly in hams of this kind. The organisms listed by Jensen *(9)* were formerly encountered during the long brine cure; Southern hams are generally cured with dry cover. Boyer *(3)* and Jensen and Hess *(10)* state that spore-forming anaerobes are the chief, if not the sole, organisms of taint. *Clostridium putrefaciens*, *Cl. histolyticum*, *Cl. sporogenes*, and *Cl. tertius* have been isolated and identified. That numerous organisms may develop in hams without spoilage, and in fact may even be contributory to the development of flavor, is indicated by Jensen *(9)* and also by Hunt, Supplee, Meade, and Carmichael *(7)* in studies of both pickle-cured and dry-cured hams. Of these, *Clostridium putrefaciens* is capable of growing at curing room temperatures, and has been widely acclaimed as the agent of taint in hams of this region.

PROCEDURE AND OBSERVATIONS

Nature of Taint or Sour. Of the several types of internal spoilage developing in hams during and after cure, loin or flesh sours are dominant. The spoilage area is initiated at, and frequently localized in, the flesh just above the stifle joint, on the inner or body side. Impression slides made of freshly cut tissue and stained with Barlow's stain usually reveal large, spore-forming rods. In many instances, the flesh in this area has undergone no visible alteration, but as taint becomes more pronounced the tissue becomes progressively more friable, with the light red pigment fading. In advanced stages the tissue softens to the point where it is butyrous. The pH is usually higher than that of comparable areas in sound hams, sometimes being as high as pH 7.2.

Frequently, the taint is so slight as to escape the trier, and is detected only after the ham has been cut or prepared. Most often, tainted hams are fairly large, 16 to

[a] In cooperation with the Food Processing Section, Tennessee Valley Authority. This investigation is part of a federated research program under the auspices of the Authority.

[b] Information obtained from Mr. R. D. Haynes of the Tennessee Valley Authority. This is the estimated annual loss due to all causes.

25 pounds; smaller hams become obnoxiously gassy, and less often tainted without gas. The odor ranges from "cheese like" to butyric, and is distinctive of clostridial development.

It is not often that cultures can be obtained even a short distance from the focal point of spoilage, yet volatile products will have diffused into major portions of the ham, rendering most or all of it unfit for use by the discriminating consumer. Isolations have been made from bone marrow only occasionally.

Isolation and Identification of Organisms. The medium for isolation and maintenance was a modified liquid consisting of equal parts of liver infusion and Difco brain-heart infusion, to which was added 1% tryptone, 0.5% glucose, and 0.1% yeast extract. A small amount of dried infused liver was added to each tube of medium. For routine studies, and where clostridia were indicated in isolation, tubes were heated to 80°C. (176°F.) for 10 minutes. Other media widely used for anaerobes of animal origin did not support growth as well. Routine inoculum was approximately 0.5 g. of ham or 0.2 ml. of liquid culture.

Freshly cut meat surfaces were seared either with the flame of a Bunsen burner or with hot metal, and portions of meat were removed following a cut into the sterilized area.

Identification was made according to Bergey's Manual *(2)*. Seven species, with several variants, were obtained as follows: *Clostridium bifermentans, Cl. mucosum, Cl. sporogenes, Cl. parabifermentans, Cl. septicum, Cl. paraputrificum,* and *Cl. putrefaciens.* The latter organism was isolated twice from approximately 100 ham samples.

Effect of Curing Ingredients Upon Growth of Anaerobes. Tanner and Evans *(12)* and Evans and Tanner *(5)* have reviewed the literature in regard to the effects of NaCl, $NaNO_3$, and $NaNO_2$ upon organisms and meats in cure, and have presented their own studies on the effects of these compounds upon several cultures of *Clostridium botulinum, Cl. sporogenes,* and *Cl. putrificum.* Their results show clearly that the concentrations of curing ingredients, at levels customarily encountered in curing, alone or in combination, are insufficient to prevent growth of the organisms, or, where applicable, production of toxin. Stumbo, Gross, and Vinton *(11)* found that the resistance of a putrefactive anaerobe to heat was not altered by the addition of 3½ lb. NaCl, 2¾ oz. $NaNO_3$ ½ oz. $NaNO_2$, and 1 lb. sugar per hundred pounds of meat.

To ascertain the limiting concentrations of NaCl and nitrate, additions of each were made to the basal medium. After heat shock, tubes were incubated at 30°C.(86°F.) for 7 days. Results are shown for *Clostridium septicum* only (Table 1). Similar results were obtained with other organisms. Cultures of *Cl. putrefaciens* were inadvertently lost, and are not included in these studies.

TABLE 1

The Effect of KNO_3 and NaCl on the Growth of Cl. septicum in Basal Beef Liver-Brain-Heart Infusion Medium

NaCl, %	KNO_3, %		
	0.00	0.05	0.10
6.0	Excellent	Excellent	Excellent
8.0	Fair	Good	Fair
10.0	None	None	None

At a level of 6% NaCl *Cl. septicum* grew excellently with or without 0.1% $NaNO_3$ (0.0615% nitrate ion). The extent of growth decreased at 8% NaCl, and neither this nor other cultures grew at a concentration of 10% NaCl. None of the cultures reduced the nitrate.

Presumably, the nitrite ion in combination with higher levels of salt is toxic to clostridia. This is demonstrated in Table 2, in which it is shown that in the basal medium with 0.05% KNO_2 (0.027% nitrite ion) all cultures grew and 8 of 10 at twice this concentration of nitrite. Likewise, all grew well with 4% NaCl, and all but one at 6%. When nitrite was added to the 4% NaCl, however, one organism, a strain of *Cl. mucosum* differing from the others only in its ability to form a curd in milk and to

give alpha hemolysis on blood, grew. It was stopped at 6% NaCl with added nitrite. In this and subsequent tables, results are given in terms of percent of organisms tolerating given concentrations, since at borderline concentrations individual cultures grow sporadically.

TABLE 2

The Inhibitory Effect of NaCl and KNO_2 upon Anaerobes Grown in Basal Medium, as Shown by Percent of Cultures Growing

NaCl, %	KNO_2, %		
	0.00	0.05	0.10
0.0	100	100	80
4.0	100	10	10
6.0	90	0	0

Effect of pH of Medium and Added Curing Ingredients. Ingram *(8)* reviewed the British work which demonstrated a close correlation between pH and taint. The lower the pH of freshly killed pork, the less is the chance for spoilage. This has been confirmed by Gibbons and Rose *(6)* and Winther and Nielson *(13)*.

Varying lots of medium were adjusted to pH values ranging from 5.5 to 7.2 (after sterilization) and inoculated and incubated as usual. All cultures grew, the extent of growth being moderate to good, even at the lowest level of pH. Lower levels were not included, since the lowest pH commonly reported for muscle tissue is 5.4, *(4, 13)*.

NaCl, added to the medium at varying concentrations up to 6%, failed to prevent growth, although at the greatest concentration at pH 5.8 and 5.45 there was some inhibition. Growth was poor at 7% NaCl at all levels of pH ranging from 5.5 to 6.4.

Marked inhibition was obtained with the addition of nitrite. At a level of 5% NaCl and 0.03% KNO_2 (0.01575% nitrite ion) 80% of the cultures grew at pH 6.3, but only 20% grew at pH 5.5. Whether results would have been different had nitrate been used is not known. Jensen *(9)* cites (p. 14) Grindley, MacNeal, and Kerr to the effect that in acid solutions of brine small amounts of nitrate exerted a marked inhibitory effect upon bacteria. This action was ascribed in part to the reduction of nitrate to nitrite by reducing substances present.

Effect of Low Temperature Upon Survival. Attempts are made to hold the temperature of the curing rooms as close to 4.4°C.(40°F.) as possible. To determine whether the organisms involved in this study would grow at this temperature, a full range of concentrations of NaCl and KNO_2 was added to the basal medium at pH 6.3 and 5.5. After inoculation the tubes were held in a curing room at approximately 7.2°C. (45°F.) for 8 weeks. None, including the controls, showed growth on visual examination. These tubes were then incubated at 32°C.(89°F.) for 7 days (Table 3). Beyond a

TABLE 3

The Inhibitory Effect of NaCl, KNO_2 and pH upon Anaerobes when Stored 8 Weeks at 7°C.(45°F.), as Shown by Percent of Cultures Growing when Incubation Continued at 32°C.(89°F.)

NaCl, %	KNO_2, %		
	0.01	0.03	0.05
	Percent of cultures growing at pH 5.5		
1.0	80	30	50
2.0	60	70	30
3.0	60	60	70
4.0	60	30	30
	Percent of cultures growing at pH 6.3		
1.0	80	50	10
2.0	70	10	10
3.0	80	30	10
4.0	80	10	10

concentration of 0.01% KNO_2 (0.00525% nitrite ion) an inhibitory effect is more noticeable with increasing concentration of NaCl. At pH 6.3, only one organism, at each concentration, tolerated 0.05% KNO_2.

At the end of cure, hams are washed, then dried and smoked for 24 hours or longer at elevated temperatures. To determine whether prolonged exposure at a higher temperature has a lethal or inhibitory effect, inoculated tubes of medium containing 0, 2, and 4% NaCl were held at 48°C.(118°F.) for 7 days, and examined for growth (Table 4). Eighty percent of the cultures in the salt-free medium grew, but none in the presence of 2% NaCl. The tubes were removed to the 32°C.(90°F.) incubator and observed after 7 days. The remaining cultures in the salt-free medium grew, as did 80% of those in the tubes containing NaCl.

From these results, it may be concluded that organisms surviving the curing period may not be killed during the drying and smoking period. Actually, our temperature of incubation is strenuous when compared to practical conditions, for although meat may be held in the smoke house at an air temperature of 51.5° to 54.4°C.(125° to 130°F.), the internal temperature is approximately 47.8°C.(118°F.) at the end of 24 hours. Consequently, the temperature at the customary focus of spoilage is well within the range permitting germination and growth of the anaerobes for a long period of time. The question of spoilage occurring while hams are in the smoke house is frequently raised, but because of many complex factors is at present unanswerable. It is the subject of another investigation.

TABLE 4

The Inhibitory Effect of NaCl on Anaerobes Incubated in Basal Medium at 48°C.(118°F.), and Ability to Survive as Shown by Continued Incubation at 32°C.(89°F.)

NaCl, %	Percent growing at 48°C. (118°F.) in 7 days	Percent growing on continued incubation at 32°C.(89°F.) for 7 days
0.0	80	100
2.0	0	80
4.0	0	80

DISCUSSION

The results obtained in this work tend to clarify to some extent the factors leading to taint in country-style hams, yet raise additional problems for investigation. Many plant operators and workers associated with them believe that much of the spoilage develops during the curing period. This period is determined by the weight of the meat, at the rate of two days per pound. Significantly, only one species isolated in this work may be considered a psychrophile, *Clostridium putrefaciens*. It was obtained twice from nearly 100 hams submitted for examination. None of the other organisms grew over a period of 8 weeks at a temperature of 7.2°C.(45°F.), which is a higher temperature than is normally encountered in the curing plants. It appears probable that the bacteria develop during the chilling period, which for farm-slaughtered animals is not always ideal, or after the hams emerge from cure.

The effect of curing ingredients upon clostridia are, on the whole, misleading with respect to dry cured hams. Under the conditions of Jensen's researches, reported in his textbook on meats *(9)*, hams were pumped. This means that a brine solution was introduced into the circulatory system, and then the ham was immersed in brine. The majority of southern hams are cured by applying dry curing mixture to the surface at the approximate rate

of 8 lb. NaCl, 2 lb. sugar, and 2 oz. nitrate per 100 lb. of meat. The curing ingredients are taken up independently of each other and move slowly into the meat. Much of the salt is lost through "drip," brine running from the meat during the first few days in cure. NaCl is not uniformly dispersed throughout the ham, or even throughout the same muscle, as is shown by the work of Beasley and Carroll *(1)*. This fact, together with the slow rate of heat loss of the carcass in the chill room, may account for the frequent localization of spoilage.

Our analyses of hams approximately 4 months out of cure have shown that the content of NaCl in the center muscle is in the vicinity of 4 to 5%, and the concentration of nitrite ion at around 0.0003 to 0.001% in the affected muscle. These concentrations are obtained only after a long period of time, and in any event are below those necessary for inhibition of growth of the anaerobes. Quite definitely, the effect of curing ingredients may be additive to other known inhibitory factors as pH and dehydration in preservation, and possibly even additional unknown factors. The latter point is made advisedly, for in one attempt, a number of cultures isolated from tainted ham failed to grow in a medium prepared according to Yesair's formula for pork infusion, using cured ham as the meat.

SUMMARY AND CONCLUSIONS

Taint develops initially in hams above the stifle joint and in muscle in the inner side of the ham. In cases of mild taint, organisms are isolated from this area only, and from this area volatile products diffuse throughout the meat, rendering the entire piece undesirable.

Isolates from tainted Southern country-style hams submitted by locker plant operators were identified as members of the genus Clostridium, and are *Cl. bifermentans, Cl. mucosum, Cl. sporogenes, Cl. parabifermentans, Cl. septicum, Cl. paraputrificum,* and *Cl. putrefaciens.* Of these, only the last organism is capable of growing at psychrophilic temperatures.

The limiting concentrations for growth of curing ingredients are above those encountered in meat at the end of 4 months' storage period, and 5½-6 months after slaughter. The curing ingredients themselves in quantities absorbed by the meat do not prevent the growth of the anaerobes.

Results indicate that a material reduction in the number of hams having taint may be made by proper attention to the control of temperature, that is, cooling and maintaining hams at the temperature recommended by the meat industry.

Results indicate that the majority of organisms responsible for taint fail to grow in properly chilled and refrigerated meat. From this one may conclude that the incidence of taint may be materially reduced by proper attention to the above factors.

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ON THE DEVELOPMENT OF OFF-FLAVOR DURING THE STORAGE OF FROZEN RAW PEAS*

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It is well known, that to secure high quality frozen peas it is necessary to blanch this vegetable before placing it in storage and that unblanched peas held in frozen storage develop undesirable off-flavors that are frequently described as "hay-like" in character *(4)*.

The present investigation was started with the object of determining the cause of these off-flavors.

EXPERIMENTAL PROCEDURE

Preparation of Sample. Thomas Laxton peas were grown on the Experiment Station farm during the 1945 season and harvested in the stage that would yield a fancy frozen product. They were vined mechanically, washed, and hand-picked to eliminate bruised and otherwise injured peas. They were then divided into two lots, one of which was packed without further treatment. The other lot was blanched by immersion in boiling water for sixty seconds, cooled in cold water, drained and packed. Both lots were frozen, transferred to storage at −17.8°C.(0°F.) and kept under these conditions for five years. Other lots of this same variety were similarly prepared for immediate analysis during the 1950 season.

Analytical Methods. For the general analytical work, 150 g. of the peas were placed in a Waring blendor with an equal weight of water and mixed four minutes. This slurry was used for the following determinations: total solids, total nitrogen, reducing sugars, sucrose, and starch. Samples for the determination of total solids were taken to dryness on a steam bath and then placed in the vacuum oven at 70°C.(158°F.) for 48 hours. Samples for the determination of total nitrogen were transferred to Kjeldahl flasks, and the nitrogen determined by the Gunning method, modified to include nitrate nitrogen by the addition of salicylic acid to the sulfuric acid *(2)*. The sugars were determined by the Munson-Walker gravimetric method *(2)*. A colorimetric method for starch was employed *(7)*.

Lipid matter was extracted from the peas in the following manner: First, 250 g. samples of peas were lyophilized. Lyophilization was chosen in preference to oven drying in order to minimize deteriorative changes in the peas prior to analysis. This procedure netted about 40 g. of peas which were then ground to a powder. The powder was extracted in all-glass Soxhlet equipment using pure, peroxide-free, redistilled, anhydrous ethyl ether as solvent. The solvent was removed from the lipid matter by evaporation at room temperature. Finally the crude lipid residue was dried over P_2O_5 overnight in a vacuum desiccator. This procedure yielded enough lipid matter so that microdeterminations of the peroxide number, iodine number, and acid number could be made.

The methods used for the determination of peroxide number, iodine number, and acid number were micromodifications of existing methods. The sample size was scaled down to 20 to 40 mg. in the case of peroxide number, acid number, and iodine number samples. Similar reductions were made in the quantities of the various reagents used in these methods. All of these determinations were made in small (25 mm. x 110 mm.) test tubes, equipped with ground glass stoppers. The lipid material was accurately weighed directly into the test tubes.

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Iodine numbers of the lipid fractions were determined by a micromodification of the Hanus iodine number method, similar to that of Ruziczka *(8)*. Twenty to forty mg. of the lipid was dissolved in 1.0 ml. of chloroform and 2.0 ml. of Hanus iodine monobromide solution *(2)* was added. The test tube was then stoppered and a few drops of glacial acetic acid were placed on the ground glass surface to form a liquid seal. The tube was shaken by rotating gently for one-half a minute and placed in the dark with occasional shaking. Exactly 30 minutes after the addition of the Hanus solution, 1.0 ml. of 15% KI was added, the stopper replaced, and the test tube shaken for one-half a minute. Ten ml. of distilled water was introduced into the test tube, taking care to wash any traces of iodine from the stopper into the tube. The sample was then immediately titrated with standard 0.1 *N* sodium thiosulphate to a pale straw color, six drops of 1% starch solution were added and the titration completed to the disappearance of the blue color. For each determination, two blanks were included, employing all of the reagents but omitting the lipid.

Acid numbers were determined by a micromodification of the standard A.O.A.C. procedure *(2)*. Twenty to forty mg. of lipid matter was dissolved in 5.0 ml. of 95% alcohol neutralized to phenolphthalein, the tube was heated in a water bath nearly to boiling, one drop of phenolphthalein solution was added, and the sample was titrated with standard 0.01 *N* NaOH, and the acid number calculated in the usual manner.

Peroxide numbers were determined by a micromodification of a method similar to that of Wheeler *(10)*. Twenty to forty mg. of lipid matter was dissolvd in 1.0 ml. of chloroform-glacial acetic acid mixture, prepared by adding in a 250 ml. graduated cylinder 150 ml. of glacial acetic acid to 75 ml. of $CHCl_3$. The mixture was placed in a 250 ml. glass stoppered bottle, and shaken vigorously. To this was added 0.01 ml. of saturated KI solution and the test tube was shaken for exactly one minute. Then 1.0 ml. of distilled water was added, and the sample was titrated immediately with 0.001 *N* sodium thiosulphate to a pale straw color, six drops of 1% starch solution were added and the titration completed to the disappearance of the blue color.

The chlorophyll and beta carotene content of the peas used were also determined. The procedure for the chlorophyll determination was taken from the work of Mackinney *(5)* on the absorption of light by aqueous acetone (80%) extracts of chlorophyll. The concentrations of chlorophyll a and b were determined by measuring the density of 80% acetone chlorophyll extracts in a 1-cm. cell with a Beckman spectrophotometer at 663 and 645 mμ. The optical density values at these two wave lengths were substituted into the simultaneous equations developed by Arnon *(1)*.

Carotene was determined by the "foaming mixture" method of Moore and Ely *(6)* followed by purification of the extract by partition on a chromatographic column of 1:3 MgO and diatomaceous earth (Hyflo Supercel, Johns-Manville Co.) *(9)*.

TABLE 1

Effect of Storage on Major Components of Frozen Thomas Laxton Peas.
(All Samples Analyzed July, 1950[b])

Year harvested	Storage, years	Treatment	Total solids	Reducing sugars (glucose)	Sucrose	Starch	Total nitrogen
			%		%	%	%
1950	0	Unblanched	19.61	Negative	26.72	15.68	4.42
1950	0	Blanched	17.34	Negative	25.82	15.73	4.44
1945	5	Unblanched	21.26	Trace	26.64	18.85	4.59
1945	5	Blanched	16.85	Negative	22.84	18.12	4.62

[b]All results expressed on the dry weight basis.

RESULTS

Analyses were made of the raw and blanched samples from the 1945 series following removal from storage in 1950, and from samples of the peas harvested in 1950 immediately after preparation (Table 1). Data are not available for the 1945 series immediately after processing. The

results of the sugar and starch determinations indicate that little change took place in these fractions during the storage period. The data also indicate that no gross changes had occurred in the overall nitrogen content. Possible changes among the various nitrogenous components which might have taken place during storage of the unblanched peas, and which may have led to the presence of volatile amines, were investigated in the following manner:

A mixture of 180 g. of frozen unblanched Thomas Laxton peas (held in storage for five years) and an equal weight of water were whipped in a Waring blendor for 4 minutes and placed in a balloon flask. An all-glass distillation apparatus was employed. The balloon flask was connected by means of a West condenser to a receiving flask cooled in an ice bath. This flask was connected to a series of three flasks immersed in a cooling mixture of Methyl Cellosolve and dry ice. A pressure of 20 mm. of mercury was maintained in the system with an aspirator. A very slow stream of nitrogen was run through the system during the operation. The flask containing the pea slurry was heated by immersion in a water bath held at 40 to 50°C.(104 to 122°F.). After five hours of operation no volatile off-flavors could be detected in any of the traps. This suggests that volatile amines were either absent or were of minor importance in contributing to the off-flavors found in unblanched frozen peas after long periods of frozen storage.

The lipid fraction was next investigated. The lipid matter was prepared by Soxhlet extraction of lyophilized material from blanched and unblanched samples of peas held in frozen storage for five years and also from fresh samples of the 1950 crop. These lipid extracts were analyzed for peroxide number, iodine number, and acid number (Table 2).

TABLE 2

Peroxide Number, Iodine Number, and Acid Number of Crude Lipid Material Extracted from Thomas Laxton Peas.

(All Samples Analyzed July, 1950)

Year harvested	Storage, years	Treatment	Peroxide number	Iodine number (Hanus)	Acid number
1950	0	Unblanched	Negative	98.0	22.6
1950	0	Blanched	Negative	99.2	23.0
1945	5	Unblanched	27.3	88.7	97.2
1945	5	Blanched	Negative	98.0	18.6

When lipids become rancid either through hydrolytic or oxidative action, certain characteristic changes can be observed in the various fat "constants." Among the changes noted in oxidative rancidity are an increase in peroxide number and acid number and a decrease in the iodine number. The most reliable of these three criteria for the detection of oxidative rancidity is the increase in the peroxide number. The peroxide number showed a value of zero in the lipids from the 1950 crop of peas as compared to a value of 27.3 in the lipids from the stored unblanched peas of the 1945 crop. The lipid material from unblanched peas which had been stored at −17.8°C.(0°F.) for five years also gave evidence of rancidity in the other two tests, namely, a higher acid number and a lower iodine number than were found in the lipid material from the 1950 crop.

The residue remaining after ether extraction of the unblanched peas of the 1945 crop was found to have a flavor that was suggestive of that of a cereal, with a slight flavor of pea. Rancid flavor was completely lacking when it was tasted dry, or after it had been cooked with water. The

taste of the extracted lipid material from these unblanched peas stored for five years at —17.8°C.(0°F.) was decidedly rancid. The odor, too, of this lipid material was typically that of a rancid fat, all of which corroborated the results of the chemical analyses, i.e., high peroxide number, lower iodine number, and high acid number. On the other hand, the lipid extracted from the blanched peas stored for five years at —17.8°C.(0°F.) showed no taint of rancidity. The extracts prepared from blanched and unblanched peas of the 1950 crop immediately after harvest likewise showed no evidence of rancidity. These findings show that rancidity in the lipid fraction is a dominant factor in the development of off-flavor in frozen unblanched peas.

TABLE 3

Chlorophyll and Beta Carotene Content of Thomas Laxton Peas.
(Analyzed July, 1950[c])

Year harvested	Storage, years	Treatment	Total chlorophyll, mg./100 g.	Beta carotene, micrograms /100 g.
1950	0	Unblanched	40.05	211.4
1950	0	Blanched	57.18	299.7
1945	5	Unblanched	23.40	93.7
1945	5	Blanched	60.75	240.4

[c]All results expressed on the dry weight basis.

Pigments. It has been observed on numerous occasions that unblanched vegetables show deterioration in color during storage at —17.8°C.(0°F.). It was already known that the carotene content of certain unblanched vegetables including peas decreases during storage at this temperature *(11)*. An investigation was made of the content of chlorophyll and beta carotene in blanched and unblanched peas of the 1945 crop after five years of storage at —17.8°C.(0°F.) and of similar samples from the 1950 crop, analyzed immediately after harvest. The results of this work are given in Table 3. It is obvious from the values in Table 3 that a loss of pigment has occurred in the unblanched peas held at —17.8°C.(0°F.) for five years, which loss did not take place in the blanched peas. Actually, when the method and calculations of Arnon *(1)* were employed, the figures for chlorophyll a and b were found to be as follows (mg./100 g. dry wt. basis): 1945 crop, unblanched peas, chlorophyll a, 12.18, chlorophyll b, 11.23; blanched peas, chlorophyll a, 38.09, chlorophyll b, 22.70. However, as is suggested by the work of Mackinney *(5)*, it is likely that these figures for the individual chlorophylls are not entirely reliable because of the probable presence and interference of pheophytin, but are reported here for comparative purposes. It is interesting to note that based on these figures, the greater loss occurred in chlorophyll a, the more labile one of the two forms.

DISCUSSION

The results presented in this paper show that rancidity of the lipid material is one of the primary, if not the principal cause of the development of off-flavor in unblanched peas stored at —17.8°C.(0°F.). This conclusion was reached as a result of the following information. The per-

oxide number of the crude lipid matter extracted from the unblanched peas after storage for five years at —17.8°C. was high and the acid number was high also. Contrasted with this was the almost negative peroxide number found for the crude lipid extracted from the blanched peas of the same original lot, stored under the same conditions. The acid number of the lipid matter obtained from the blanched peas was about one-fifth of that of the lipid matter obtained from the unblanched material. Further, the iodine number of the lipid matter obtained from the unblanched peas was lower than that of the crude lipid matter from the blanched peas; such a reduction in iodine number is another criterion of rancidity. Tests made on the crude lipid matter, prepared from blanched and unblanched lyophilized peas of the 1950 season of the same variety (Thomas Laxton) and run immediately after harvesting and processing showed negative peroxide numbers on both blanched and unblanched lots. The iodine numbers of the crude lipid material from blanched and unblanched peas of the 1950 crop were essentially equal, and the same was true for the acid numbers of the respective lipid fractions. These lipids extracted from the peas of the 1950 crop showed no signs of the rancidity which characterized the lipid fraction of the unblanched peas after five years of storage. This indicates that the blanched samples underwent no deterioration in the lipid fraction during five years of storage at —17.8°C.

It is interesting to note that Burton *(3)* came to the conclusion that off-flavor in mashed potato powder was probably caused by oxidative rancidity of the fat. However, his conclusions were not based upon direct chemical analysis of the lipid material.

SUMMARY

Blanched and unblanched Thomas Laxton peas were lyophilized after five years of storage at —17.8°C.(0°F.), and the lipid material was extracted with peroxide-free anhydrous ethyl ether. Similar ether extracts were prepared from blanched and unblanched fresh peas of the same variety immediately after harvest.

Experimental results based on peroxide number, iodine number, and acid number, as well as on taste and odor, have shown that rancidity of the lipid material is one of the primary causes, if not the principal cause of the development of off-flavor in unblanched frozen peas during storage at —17.8°C.(0°F.).

No changes of significance were observed in the reducing and nonreducing sugar fractions and in the starch fraction. Thus it appears that these components of the carbohydrate fraction do not contribute to the development of off-flavor during the storage of frozen unblanched peas. No changes were observed in total nitrogen content, such as would have occurred had ammonia or volatile amines or other volatile nitrogenous compounds been evolved during storage. There was a considerable loss of chlorophyll and carotene during the protracted storage of frozen unblanched peas.

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MICROBIAL CHANGES IN REFRIGERATED LIVER SAUSAGE*

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Relatively little information is available on the bacteriology of cured sausages. Jensen *(3)* discusses the subject but speaks mainly of the part played by bacteria in producing discolorations and surface slime. He says that the bacteria in inspected sausage are not numerous and most of them belong in the genera *Pseudomonas, Achromobacter, Proteus, Micrococcus, Chromobacter, Bacillus, Serratia, Flavobacterium,* and *Lactobacillus.*

Liver sausage itself has received even less attention from bacteriologists than have certain other types. Handlers of this product have always considered it to be perishable and have been forced to insure its rapid movement to the consumer or accept loss from discoloration, surface spoilage, or excessive dehydration. Spoilage caused by microorganisms growing in the sausage has rarely if ever been reported because of the relatively short storage life of the product.

The use of artificial casings in recent years has greatly reduced the rate of water loss, minimized surface spoilage, and lessened chances for color changes by reducing the rate of oxygen absorption. The resulting longer storage life of the product presumably could allow growth of cold tolerant anaerobic or facultative organisms that survive the cooking process and thus permit types of spoilage not previously encountered. The principal aim of this study was to see what if any changes in the microbial flora of commercial liver sausage occurred during prolonged storage under good conditions of refrigeration.

Much of the liver sausage now produced in artificial casings is stuffed and cooked in Visking, a transparent cellulose derivative made by the Visking Corporation, Chicago, Ill. Because the loss of water through this film is appreciable, and perhaps for other reasons, the finished sausage is placed in another transparent envelope of a plastic film less permeable to water vapor and gases than is Visking. The commercial braunschweiger liver sausage used in this work was stuffed in Visking, then covered with a single layer of 140 gauge Pliofilm, a rubber hydrochloride product of the Goodyear Tire and Rubber Co., Akron, Ohio.

METHODS

Because it was anticipated that differences might exist in the microbial flora at different places in a liver sausage, samples were taken from three zones or areas. If one considers a cross section of the sausage, zone A represented the center, zone B was a one-half inch wide strip midway between the center and the outside, and zone C was the one-half inch wide layer adjacent to the casing. These areas are referred to hereafter as zones A, B, and C.

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Before sampling, the outer casing was stripped back, the inner casing cleaned with alcohol and the sausage cut through with a sterile knife. For zone A, a single plug about four inches long was taken with a sterile cork borer having a diameter of one-half inch. Ten grams of this was used as the sample. For zone B, five plugs were taken as for zone A, but they were distributed equally around the circumference of the zone. Two-gram portions from each plug were pooled to make the 10-gram sample. For zone C, a sterile cheese trier was used to take 10 plugs spaced equally around the periphery of the sausage. The cheese trier was used to include as much of the surface adjacent to the casing as possible. One-gram portions from each plug were combined to make up the 10-gram sample. For all plugs the portion most distant from the cut surface of the sausage was used to minimize contamination carried through by the cutting knife.

The 10-gram sample from each zone was blended for one minute with 90 ml. of sterile water in a sterile Waring blendor bowl. Appropriate additional dilutions were made in sterile water and portions cultured in triplicate as follows:

1. To detect mesophilic aerobic and facultative organisms: Difco tryptone glucose extract agar plates incubated at 37°C.(98.6°F.) for 2 days.
2. To detect mesophilic anaerobic and facultative organisms: BBL thioglycollate medium (Brewer-modified) containing 1.5% agar, poured in plates and incubated anaerobically at 37°C.(98.6°F.) for 4 days.
3. To detect organisms able to grow at low temperatures: Difco yeast dextrose agar shake cultures sealed with paraffin and incubated at 5°C.(41°F.) for 30 days.

After incubation the colonies were counted and those in the highest dilutions subcultured in thioglycollate broth. Characteristics of the resulting isolates were observed by conventional methods *(2)*. The method developed by Watson *(5)* for Swiss cheese was used for pH determinations.

RESULTS

The Microbial Content of Freshly Made Sausage

As a preliminary to actual storage trials several experiments were run to determine the extent of variation in numbers of organisms in different samples of sausage. Such information was necessary for interpretation of changes that might occur later during storage.

Numbers of Organisms in Different Samples of the Same Sausage. Commercial liver sausages approximately 2.5 inches in diameter and 24 to 27 inches long were divided into five equal portions. Zones A, B, and C were sampled from each portion, thus making a total of 15 samples per sausage. All were plated in tryptone glucose extract agar (TGEA) and thioglycollate agar (TA) but not in yeast dextrose agar (YDA) because earlier trials had shown that the numbers of organisms capable of growing at 5°C.(41°F.) were insignificant in freshly made sausage (usually less than 10 per gram).

Table 1 shows the results for four sausages representing different production lots from the same packing plant. As can be seen, no part of either sausage showed consistently higher or lower counts than did other parts, and in general the counts were uniform for all samples from a single sausage. In later trials an occasional sausage was found with considerably fewer organisms in the outer zone than in the center. This was true only in lots having a relatively high initial count of aerobes and, since it was exceptional, it probably means that an occasional sausage is heated to a higher temperature than the others because of its location in a hotter part of the cooking tank.

Numbers of Organisms in Different Sausages from the Same Production Lot. Ten sausages were chosen at random from one lot and samples were

plated in TA and TGEA. Table 2 shows that the flora of sausages from the same lot, like that of different samples of the same sausage, was relatively homogeneous. The counts in Table 2 represent averages of the values for zones A, B, and C.

TABLE 1

Counts of Mesophilic Organisms in Freshly Made Liver Sausage

Sausage No.	Zone	Counts per gram in									
		Thioglycollate agar (anaerobic)					Tryptone glucose extract agar (aerobic)				
		I[b]	II	III	IV	V	I[b]	II	III	IV	V
1	A	160	240	120	600	780	.....	6,400	15,000	12,000	19,000
	B	150	260	140	1,000	340	2,700	11,000	9,200	11,000	18,000
	C	120	180	700	370	280	1,400	7,200	4,500	6,200	10,000
2	A	370	550	520	720	660	4,700	5,100	2,300	2,700	2,400
	B	610	600	460	670	610	5,100	3,800	3,400	3,500	2,800
	C	580	520	710	650	970	3,600	2,300	2,000	2,000	2,100
3	A	90	350	340	130	130	330	400	710	460	450
	B	100	160	130	240	30	330	1,000	1,200	620	390
	C	130	200	160	150	220	290	330	330	900	270
4	A	220	250	180	200	160	260	320	210	300	230
	B	150	160	200	200	170	260	150	280	280	330
	C	210	230	160	250	230	340	280	240	320	330

[b] Roman numerals refer to the five portions into which each sausage was divided.

TABLE 2

Plate Counts from Sausages of the Same Lot

Sausage No.	Thioglycollate agar	Tryptone glucose extract agar
1	390	12,000
2	260	10,000
3	550	8,700
4	440	5,800
5	590	7,800
6	940	11,000
7	200	8,600
8	480	8,100
9	440	8,800
10	260	10,000

Numbers of Organisms in Sausages from Different Production Lots

The results in Table 3, as well as those in Table 1, show that the numbers of anaerobic organisms were uniformly low in all of the lots of liver sausage examined. The numbers of aerobes, on the other hand, were either a few hundred per gram or several thousand. When the count was high, the predominant organisms always were micrococci; when it was low, aerobic spore formers were most numerous. Presumably the sausage mix always contained considerable numbers of micrococci and relatively fewer spore formers. Small variations in the cooking temperature from one lot to another could account for the observed presence or absence of micrococci in the initial flora and thus affect the counts on the freshly made product.

These variations are of no consequence, however, because the majority of these organisms were strict aerobes and could not grow in sausage for lack of air.

Changes in the Microbial Content of Sausage During Storage

Four large commercial sausages from a single production lot were stored in a household mechanical refrigerator at 5°C.(41°F.) and sampled at intervals of approximately 1 week. After a sample was taken the cut end of the sausage was trimmed smooth with a sterile knife, a sheet of sterile Pliofilm was tied over the surface and the end of the sausage was dipped in melted paraffin. The next sample was taken 4 inches from the sealed end. One sausage sufficed for 3 samples.

TABLE 3

Plate Counts from Sausages of Different Lots

Lot No.	Thioglycollate agar	Tryptone glucose extract agar
1	360	9,500
2	610	3,200
3	170	530
4	200	280
5	50	250
6	460	8,000
7	100	5,800

The results in Table 4 show bacteria counts and pH values. The freshly made sausage contained only a few hundred organisms per gram and these were mostly spore forming bacteria that grew only in the aerobic plate cultures. Their numbers remained unchanged throughout the experiment. Growth was first observed with certainty in the outer zone C on the 25th day of storage and it continued until the counts reached several millions per gram at 39 days, with little change thereafter. A gradual but steady decrease in pH accompanied the bacterial growth.

Although the preliminary experiments showed close agreement between numbers of organisms in different parts of a single sausage as well as in different sausages from the same lot, it is evident from these results that no such close agreement was obtained after organisms grew in the refrigerated product. It must be assumed that small differences in distribution of the organisms in the freshly made sausage became magnified after growth occurred and thus caused the variations observed between samples. Nevertheless, there can be no doubt that there was considerable growth.

In the intermediate zone B, there was an apparent increase in numbers of bacteria at the 39th day but for some reason in the 61 day and subsequent samples the counts dropped back to about their original level. From the results of later experiments it is doubtful that organisms actually grew in the B zone; hence the high counts observed for the 39 to 56 day samples must be accounted for on the basis of contamination carried through the sausage by the cutting knife. Great care was taken to prevent this and that it should occur with 3 consecutive samples, then suddenly cease is difficult to believe. It is equally difficult to believe, however, that

TABLE 4

Microbial and pH Changes in Liver Sausage Stored at 5°C.(41°F.)

Zone	Sausage No.	Days of storage	Colony counts per gram in			
			TA[a]	TGEA	YDA	pH
C	1	1	50	310	10	6.55
	1	11	2,600	1,900	7,900	6.57
	1	19	60	190	3,100	6.52
	2	25	230,000	250,000	7,500	6.61
	2	32	>300,000	>300,000	360,000	6.56
	2	39	>3,000,000	>3,000,000	3,300,000	6.29
	3	47	22,000,000	2,000,000	1,100,000	6.32
	3	56	2,700,000	700,000	700,000	6.17
	3	61	320,000	280,000	320,000	6.28
	4	67	5,400,000	3,100,000	240,000	6.18
	4	75	64,000,000	63,000,000	790,000	6.04
	4	88	6,300,000	4,400,000	40,000	5.97
B	1	1	60	270	<10	6.55
	1	11	50	250	<10	6.57
	1	19	30	190	<10	6.53
	2	25	90	290	30	6.61
	2	32	50	500	200	6.67
	2	39	7,100	5,700	5,300	6.57
	3	47	13,000	16,000	6,300	6.45
	3	56	6,000	5,900	330	6.55
	3	61	140	380	330	6.51
	4	67	100	230	100	6.50
	4	75	1,100	1,800	1,900	6.42
	4	88	10	310	<10	6.38
A	1	1	40	180	<10	6.53
	1	11	40	290	<10	6.53
	1	19	60	260	<10	6.53
	2	25	70	580	<10	6.59
	2	32	20	540	<10	6.65
	2	39	80	560	<10	6.50
	3	47	110	370	<10	6.50
	3	56	60	310	<10	6.50
	3	61	70	310	<10	6.51
	4	67	10	310	<10	6.53
	4	75	10	220	<10	6.43
	4	88	<10	220	<10	6.49

[a] TA indicates thioglycollate agar; TGEA, tryptone glucose extract agar; and YDA, yeast dextrose agar used. See text for conditions of incubation.

the organisms began to grow, then died after their numbers reached a few thousands per gram. That the high counts do not represent a single atypical sausage is shown by the fact that they were found in portions of two sausages, other samples of which had low counts. Although there appeared to be a slight increase in acidity, the pH values are of no help in determining whether or not growth actually occurred because of the wide variation between samples.

There was no change in the center of the sausage (zone A) for the entire 88 days of the experiment.

After growth began in zone C there was good agreement between the counts in all three media for most of the samples, indicating that a single

kind of organism was predominant in the sausage. If this were true the organism must grow either aerobically or anaerobically at either 37°C. (98.6°F.) or 5°C.(41°F.). Organisms capable of doing this were isolated from the higher dilutions in all 3 culture media. Without exception all of the isolates from TA and TGEA plates, and most of those from the YDA shake tubes incubated at 5°C.(41°F.) were gram positive, nonspore forming, catalase negative, microaerophilic rods. They grew best at 37°C. (98.6°F.) but also grew well in 16 days at 5°C.(41°F.). Their characteristics do not agree in all details with any of the species described in Bergey's Manual *(1)*; hence identification requires further study.

A microscopic examination of the sausage during the early days of storage showed a very few yeasts. Since these organisms had not been observed in the freshly made sausage, all samples taken after the 11th day were plated in Difco potato dextrose acid agar and incubated at room temperature for 5 days. The numbers increased steadily in the outer zone C from about 1,000 per gram at 19 days to several hundred thousands per gram at 47 days and thereafter. There was no growth in the A and B zones. Some of the isolates from the YDA shake tube cultures also were yeasts, apparently the same as those that grew on the acid agar plates. None of the yeast cultures was identified because they have not been found in any other of the several storage trials. Their presence in the sausage used for this experiment must be regarded as exceptional.

During the storage period a heavy "slime" developed between the casings of the sausage. Microscopic examination showed large numbers of gram positive micrococci and a few long, gram negative rods. These organisms were not isolated and identified but morphologically they resembled those found by Lewis, Roderick, and Norton *(4)* in slime on wieners.

DISCUSSION

Because all of the sausage in a single production lot receives approximately the same treatment, it is not surprising that the microbial flora is distributed uniformly through the mass. Variations in the initial contamination of the sausage ingredients or different degrees of heating in the cooking process may account for the differences that occur from one lot to another. To be of significance in causing spoilage of refrigerated liver sausage, a microorganism must not only be fairly resistant to heat but also be able to grow at low temperatures in an environment of low oxygen tension and fairly high salt concentration (about 5% in the water). According to the results of this work the great majority of organisms that survive the cooking process are unable to meet these requirements; hence they are of no significance in causing spoilage as long as the product is properly refrigerated. Small numbers of organisms capable of growing in refrigerated liver sausage may survive the cooking process, however, and if the sausage is held too long they may contribute to spoilage.

The growth of organisms only in the outer portions of the sausage suggests the possibility of their entry through the casing after cooking. That this is not the true explanation of their presence is shown by the fact that Visking tubing is commonly used as a dialyzing membrane, hence should

be impermeable to microorganisms. Both of the films used in this study are slightly permeable to gases, however, and in time oxygen will diffuse into the sausage and raise the oxygen tension. In the present work this was suggested by the fact that a green discoloration developed beneath the casing after several weeks storage. Jensen *(3)* attributes greening of this type in liver sausage to oxidation rather than to bacterial action. Since the organisms found in this study were microaerophilic becteria and facultative yeasts, they would naturally grow more rapidly in the outer zone where the oxidation-reduction potential is higher than in the remainder of the sausage. In fact, later experiments have shown that most of the growth occurs in the outer one-eighth inch.

SUMMARY

The aerobic microbial flora of different production lots of liver sausage varied considerably, but the numbers of organisms that grew in anaerobic cultures were relatively few and fairly uniform from one lot to another. Within a single lot of sausage the distribution of organisms was reasonably uniform not only in a single sausage but also in different sausages. The predominant organisms in the freshly made product were aerobic micrococci and/or aerobic spore forming rods, neither of which grew in sausage held at 5°C.(41°F.).

After several weeks of storage, a gram positive, nonsporeforming, catalase negative, microaerophilic rod and a facultative yeast grew in the sausage adjacent to the casing. Neither of these organisms was present in detectable numbers in the freshly made sausage.

During storage a heavy "slime" of gram positive micrococci and a few long, gram negative rods developed between the layers of the casing. This growth resembled "slime" on wieners.

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INDIVIDUAL VARIATION OF THE FRUITS OF THE PINEAPPLE (*ANANAS COMOSUS L. MERR.*) IN REGARD TO CERTAIN CONSTITUENTS OF THE JUICE

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It is now generally recognized that the composition of fruits and vegetables may be influenced by environmental conditions prevailing during the period of growth and development. Soil types, cultural practices, and vagaries in climate represent a few of the factors which may cause variation in the constituents of fruits and vegetables. In addition, there may occur considerable individual variation in the composition of fruits of the same variety which are produced under apparently identical environmental conditions. An outstanding example of individual variation may be found in the fruits of the pineapple *(Ananas Comosus L. Merr.)*.

This report is an attempt to indicate the degree of variation that might be expected in individual fruits of the pineapple, at least in commercial packs, in the hope that the data may be of value to investigators who are compiling tables of nutritional values or who may be concerned with the appraisal of varieties of this fruit for use in commercial processing.

REVIEW OF LITERATURE

Information on the varietal differences in the composition of fresh pineapple fruits appears to be rather limited. Published tables of food values frequently list pineapples, fresh and canned, without reference to variety. Occasionally more specific information may be obtained by consulting the results of research with pineapples in which studies were made on the effect of some particular treatment on the composition of the fruits.

Since the results now being reported are concerned only with soluble solids (mainly sugars), acids, and ascorbic acid, the review of the literature will be restricted to these constituents.

Sherman *(6)* reported that fresh and canned pineapples contained 13-25 mg. and 10 mg. of ascorbic acid per 100 g., respectively, and that canned juice contained 5-10 mg. ascorbic acid per 100 ml. Quinones, Guerrand, and Dutcher *(5)* analyzed "cull fruits" which had "ripened in the Puerto Rican warehouse" in South Florida and found 21 mg. of ascorbic acid per 100 g. of fruit. No varietal name was given but in all probability the fruits were Red Spanish since this is the most popular commercial variety in Puerto Rico. French and Abbott *(1)* found that Florida-grown Abachi pineapples contained 35 mg. and the Sugar Loaf 40 mg. of ascorbic acid per 100 g. Some of the older analyses are now of little significance *(3)*.

Hamner and Nightingale *(2)* have made a rather extensive study of the effect of cultural and environmental factors on the composition of pineapples in the Hawaiian Islands. The authors did not identify the variety studied but since, as they have stated, only one variety is grown commercially in the Hawaiian Islands, the variety under consideration was likely Cayenne. Unfortunately no results are given for fresh fruits. In the canned juice the soluble solids ranged from 14 to 16.6 Brix, the total acid ranged from 0.47 to 1.16%, and the ascorbic acid from 2.9 to 12.8 mg. per 100 ml.

Recently Munsell, Williams, Guild, Troescher, Nightingale, and Harris *(4)* have reported on the vitamin content of three types of pineapples from Honduras, listing them under their Spanish names. The ascorbic acid content of the three varieties were given as follows: Pina, 24.6 mg.; Pina de Agua, 85.2 mg.; and the Pina de Azucar, 165.2 mg. per 100 g. of flesh.

Schappelle *(5a)*, reported sugar and acids in the juice of Red Spanish pineapples in different stages of maturity as follows: shipping green, 9.17% total sugars, 0.90% acids; turning color, 11.90% total sugars, 0.85% acids; and field ripe, 13.00% total sugars, 0.76% acids.

Watt, Merrill, Orr, Wu, and Pecot *(7)*, without reference to variety, reported the total carbohydrate contents of pineapples in raw fruit, frozen pineapples, and canned pineapple juice as 13.7, 22.2, and 13%, respectively. The corresponding values of the ascorbic acid per 100 g. were 24, 19, and 9 mg., respectively.

MATERIALS AND METHODS

Three different commercial varieties of pineapples were employed in these investigations: Red Spanish, Cayenne, and Abachi. The fruits were harvested in the "mature green" or "market ripe" stage of maturity on several commercial plantations in Florida and were shipped to Pittsburgh by railway express. This stage of maturity is characterized by a uniformly green color, with the "eyes" or fruitlets well rounded out. It does not include the fruits with a purplish color, such as are often picked for shipments to more distant places. Upon arrival in Pittsburgh the pineapples were ripened at room temperature before sampling.

An entire crate of fruits was employed in each experiment and each fruit was analyzed separately. In two instances, in which the fruits were rather large, the number of fruits per crate was 16 and 18. In all other lots there were between 25 and 36 fruits in each crate. The investigations covered a period of three years.

In preparation for analyses the pineapples were peeled, cored, and macerated in a Waring blendor. The juice was filtered through two thicknesses of cheese cloth. Total soluble solids were determined by the use of a specific gravity hydrometer. Ascorbic acid was determined by titration of the juice with 2,6-dichlorophenolindophenol. Total citric acid. Details of these methods appear elsewhere *(3a)*.

RESULTS

The ranges in total soluble solids, total acids, and ascorbic acid in the juice of the three varieties of pineapples are shown in Table 1. The Abachi

TABLE 1

Total Soluble Solids, Total Acids, and Ascorbic Acid in Florida Pineapples

Variety	Place and year of harvest	Total solids, %			Total acids, g./100 ml.			Ascorbic acid, g./100 ml.		
		Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average
Abachi	W. Boynton, 1948	13.6	7.6	9.5	0.94	0.54	0.71	65.2	38.4	51.9
Abachi	Ft. Pierce, 1949	15.3	9.1	13.2	1.00	0.55	0.86	70.8	35.7	54.8
Abachi	Miami, 1949	14.2	8.2	11.9	1.19	0.72	1.03	52.2	31.2	43.1
Abachi	W. Boynton, 1950	12.8	6.8	9.7	1.33	0.94	1.12	63.1	29.0	52.1
Cayenne	Miami, 1948	12.4	6.0	9.1	1.31	0.83	0.98	22.7	14.0	19.7
Cayenne	Miami, 1949	12.8	7.9	9.8	1.23	0.84	0.96	22.2	11.9	15.2
Cayenne	W. Boynton, 1950	12.7	9.1	11.0	0.94	0.66	0.80	16.2	10.1	12.8
Red Spanish	Pompano, 1948	11.2	8.8	10.3	1.32	0.74	0.92	23.1	11.4	17.8
Red Spanish	Pompano, 1950	16.3	11.7	13.6	1.07	0.77	0.99	23.3	9.2	17.1

and Cayenne varieties were tested during 1948, 1949, and 1950, while Red Spanish were available only during 1948 and 1950. Two lots of Abachi were sampled in 1949. Four different pineapple plantations were represented in these studies.

A considerable difference between minimum and maximum values for total soluble solids was found in the majority of the experiments. The greatest variation in soluble solids appeared in the Cayenne pineapples shipped from Miami in 1948. Although the average soluble solids content for all fruits was 9.1%, the minimum was 6.0 and the maximum was 12.4%. The Red Spanish pineapples showed the least variation in total soluble solids, the ranges being 8.8-11.2% in 1948 and 11.7-16.3% in 1950. Values for the remaining lots fell in between these two extremes. The average increase of maximum over the minimum for all lots was 64.0%.

TABLE 2

Individual Variation in Ascorbic Acid Content of Florida Pineapples (1950)

Abachi (West Boynton)		Cayenne (Miami)		Red Spanish (Pompano)	
Frequency	mg./100 ml.	Frequency	mg./100 ml.	Frequency	mg./100 ml.
1	29	3	10	1	9
1	36	2	11	2	10
2	44	5	12	1	11
1	46	2	13	1	12
1	47	2	14	4	15
1	51	1	15	4	16
2	52	3	16	1	17
2	53	....	....	4	18
2	55	....	....	2	19
4	57	....	....	3	20
2	58	....	....	2	21
1	59	....	....	1	22
1	60	....	....	3	23
1	63	....	....	....	....

Total acids also showed considerable variation, though not as great as was the case with soluble solids. The Cayenne from Miami (1948), which showed the greatest variation in soluble solids, showed the least variation in regard to total acids, an increase of the maximum over minimum of 57.8%. Greatest variation in acids was found in the Abachi variety shipped from Ft. Pierce in 1949. The maximum value for total acid in this instance was 1.00, the minimum 0.55, and the average 0.86 g. per 100 ml. of juice.

By far the greatest variation between individual fruits was to be found in the ascorbic acid content. The least varietal variation was shown by the Cayennes in 1950 in which the maximum ascorbic acid content represented an increase over the minimum of 60%. Considerable variation was shown by the Red Spanish variety shipped during 1950 when the maximum ascorbic acid was 153% higher than the minimum. The percentage of increase of the maximum over minimum ascorbic acid content for all lots averaged nearly 90%.

In order to give a clearer picture of the individual variation of pineapple fruits some of the data are presented in another manner. Table 2 shows the frequency with which the ascorbic acid values were distributed in the three varieties during 1950. Space does not permit presenting all of the data in this manner but the results of these three experiments are typical of all of them. The table has been simplified to some extent by "rounding out" the fractions of a milligram. Thus, the 22 Abachi pineapples could be grouped into 14 different categories, the 29 Red Spanish fruits fell into 13 groups, and the 18 Cayennes were distributed over 7 groups.

TABLE 3

Variability in Carefully Picked Pineapples

Variety and condition	Place and date of harvest	Total solids, %			Total acids, g./100 ml.			Ascorbic acid, g./100 ml.		
		Max.	Min.	Average	Max.	Min.	Average	Max.	Min.	Average
Cayenne (green)	Ft. Green, 7/29/42	12.8	10.3	11.7	0.89	0.53	0.78	19.5	14.3	17.4
Cayenne (turning)	Ft. Green, 7/29/42	15.1	11.6	12.8	0.85	0.63	0.72	14.3	10.7	12.1
Abachi (turning)	W. Boynton, 10/30/42	13.2	10.7	11.8	0.86	0.67	0.77	71.2	53.9	62.4

It is possible to reduce the degree of variation in pineapples by exercising extreme care in selecting the fruits in the field. On one occasion the senior author was in Florida and personally selected the fruits from the plots. These were analyzed individually and the results appear in Table 3. The three lots represent 8, 7, and 15 fruits, respectively. It is apparent that much of the variation, though not all, has been eliminated. The total soluble solids in these fruits showed a range of 23 to 30% increase in maximum over minimum. Total acids showed a little greater variation in that maxima were from 28 to 67% greater than minima. Ascorbic acid showed only slightly more variation than did total soluble solids. Maxima in the three lots were 32, 33, and 36% greater than the minima.

It must be realized, of course, that commercial pickers can not be expected to exercise the care required to select samples in this manner. It serves to show only that a certain amount of variability may result from the manner of selecting the fruits at the time of harvest.

DISCUSSION

The results of these investigations indicate the necessity for large samples in experimental work with pineapple fruits. This is especially true if samples are collected from commercial lots of fruits on the market. Production of pineapples is restricted to the tropics and it is often not practicable for the investigator to collect his own material when working with this type of fruit.

There have been occasions when research workers have reported on the nutritional value of certain fruits and vegetables and the report was accompanied by the statement that "the sample was obtained from the

local market." The results reported here serve to emphasize the amount of caution that must be exercised in collecting samples in this manner.

Pineapple pickers attempt to pick fruits in a uniformly mature green stage of maturity, basing their decision upon the color of the fruit. At the packing house the fruits are graded for size. A commercial pack of pineapples should presumably consist of fruits that are uniform in regard to both size and stage of maturity. However, matching pineapples for physiological maturity is a difficult task. No matter how uniform the green fruits appear when picked, the individual fruits may subsequently ripen at different times after being harvested.

If present means of handling pineapples could be improved in such manner as to permit shipping of riper fruits, some of these difficulties might be avoided. A pineapple picked in the "turning" stage (half-yellow and half-green) will ripen with much better quality than will a fruit that has been harvested in the mature green stage. Pineapples in the turning stage are characterized by higher percentages of sugar and lower concentrations of acids so that, even with considerable variation in these constituents, certainly a larger number of the fruits would be acceptable to the consumer.

SUMMARY

Observations were made on individual variations of pineapple fruits in regard to total soluble solids, total and ascorbic acids in the juice. The studies were conducted over a period of three years, during which time analyses were made on four lots of Abachi pineapples, three of Cayennes, and two of Red Spanish.

Each variety of pineapple showed considerable individual variation in each of the three juice constituents. Greatest range of variation was found in ascorbic acid (maximum being 60 to 150% over minimum) and the least in the total acid content (38 to 78% increase of the maximum over minimum).

Extreme care in selection of the fruits reduced the amount of variation to a certain extent, but did not eliminate it. The results indicate that a considerable degree of individual variation in the composition of pineapple fruits is to be expected and that this variation may be especially pronounced in commercial packs of these fruits.

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TOXICOLOGICAL STUDIES ON ISOPROPYL AND STEARYL CITRATES[a,b]

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In view of the increased demand for margarine in recent years, a need has developed for new sources of edible vegetable oils for hydrogenation. Since the production of soybean oil has been markedly increased in the United States within the last decade, the margarine industry has naturally turned to this oil to supplement the supply of other vegetable oils. Thus, while 87 million pounds of soybean oil were used in 1941 for hydrogenation, out of a total of 257 million pounds of all vegetable oils, the quantity of this oil employed in a 12-month period, ending June 30, 1950, was reported as 233 million pounds out of a total of 701 million pounds used by the margarine industry. In other branches of the edible fat industries, there has also been an increased usage of soybean oil.

The chief difficulty in the use of soybean oil is its property of undergoing a so-called "flavor reversion." This change results in the development of an unpleasant taste. The reversion begins in both limpid and hydrogenated oils almost immediately after deodorization. According to Martin, Schepartz, and Daubert *(20)*, Schepartz and Daubert *(30)*, and Stapf and Daubert *(32)* this reaction is associated with the production of carbonyl compounds.

Citric acid is especially satisfactory as an anti-flavor-reversion agent. However, neither citric acid nor its salts dissolve in sufficient amounts in oil to be used conveniently for this purpose.

Vahlteich, Gooding, and Neal in rotating authorship *(8, 9, 10, 11, 22, 23, 24, 35, 36, 37, 38, 39)* have recently reported the synthesis and effectiveness of fat-soluble esters of citric acid in inhibiting the flavor-reversion of soybean oil, and in stabilizing the flavor of other fatty foods. The compounds were mixed mono-, di-, and tri-esters of citric acid, containing from 3 to 18 carbon atoms in the alcohol moiety. The mono-esters were the effective agents in stabilizing oil flavor while the di-esters, particularly the higher homologues, exhibited in addition emulsifying properties.

Before the above-mentioned esters of citric acid can enjoy any widespread application in foods, it is necessary to prove that they are not toxic.

Obviously, it is not possible to test in a thorough manner all of the esters of citric acid mentioned in the patents cited. The lowest and highest homologues in the series were subjected to exhaustive toxicological and metabolic studies. The lowest homologue selected was the mixture of

[a] Supported by a grant from The Best Foods, Inc. Some of these results were reported at the joint meeting of the Northern and Southern California Sections of the Institute of Food Technologists in February, 1949.

[b] Contribution No. 275 of the Department of Biochemistry and Nutrition.

isopropyl citrate esters, predominantly the mono-ester, and the highest homologue selected was the mixture of stearyl citrate esters, predominantly the di-ester, having a melting point of 57 to 58°C. By the above it is not meant that the toxicity studies represent a blanket investigation of all homologues between the isopropyl and stearyl citrate esters. The basis of selection of the lowest homologue was because of its greatest effectiveness per unit weight in stabilizing the flavor of oils. The stearyl esters were chosen because these were far more readily soluble in oils than the lower members of the series. Indeed this same reasoning constitutes the bases for selecting these two types of esters in commercial application *(25)*.

As far as the hydrolysis products of these selected esters of citric acid are concerned, there is no danger of toxicity, even for a consumption of many times the levels required to preserve the oils. Both hydrolysis products of stearyl citrate esters are components of animal tissues, and are produced by normal metabolic processes. Not only are citrates widely distributed components of foods such as the citrus fruits and milk, but they are also intermediary products of carbohydrate metabolism in the so-called citric acid cycle *(14, 33)*. At the present time, the use of "citric acid and harmless citrates" is permitted in margarine without any specified limit, but only when used for the purpose of producing diacetyl *(6)*.

Stearyl alcohol has been found by Schoenheimer and Hilgetag *(31)* to occur in the feces of dogs; cetyl alcohol, however, was found to be a normal constituent of the feces of dogs, cats, and men. In fact, Stetten and Schoenheimer *(34)* have demonstrated that an interconversion of stearyl alcohol and stearic acid is a usual metabolic process. Moreover, stearyl stearate has been shown by Hilditch *(12)* to account for 2.5% of the total lipid in the head oil of the sperm whale.

Although isopropyl alcohol has toxicological properties at relatively high levels, no effects of this compound have been demonstrated which do not also result from the ingestion of ethyl alcohol *(16)*. Isopropanol has been shown to be approximately twice as toxic as is ethyl alcohol *(18)*. However, doses from 0.75 to 5.28 ml. of isopropanol per kg. body weight per day have been administered to rats without the development of any untoward symptoms. Administration of this alcohol over prolonged periods failed to show evidence of any delayed toxic effects *(16)*. It was likewise found that no deleterious effects on growth, pregnancy, or lactation resulted over several generations in rats given considerable quantities of isopropanol daily *(19)*.

The isopropyl citrates are dispersible in fat with some difficulty whereas the stearyl citrates are readily soluble in fat. For this reason, the first type of citric acid ester is introduced into an oil in a suitable vehicle. For this purpose, a mixture of mono- and diglycerides (1:1) of a vegetable oil is most desirable *(39)*.

The mixture of mono- and diglycerides which is used in conjunction with isopropyl citrate cannot be considered to be a substance possessing toxicity. Mono- and diglycerides occur physiologically in the digestion of fats *(7)*. Alpha-monopalmitin has been identified as a minor constituent of adrenal lipids *(29, 42)*, while Jones, Koch, Heath, and Munson *(13)* have recently reported the isolation of this monoglyceride from normal

hog pancreas. The satisfactory utilization of monoglycerides in feeding experiments has been reported by several workers. Ames, O'Grady, Embree, and Harris *(1)* have recorded that the growth and reproduction in rats fed diets containing 15 or 25% of monoglycerides, over 3 generations, were just as satisfactory as was the case when the corresponding amounts of cottonseed oil were employed. Mattson and Beck *(21)* also obtained as good growth in rats when mono- and diglycerides were incorporated in the diet as when corresponding amounts of neutral fats were used. These results are in line with unpublished results obtained in our laboratory. They also confirm the earlier experiments of Braun and Shrewsbury *(2)*, who studied the nutritional value of monostearin. At the present time mono- and/or diglycerides of fat-forming fatty acids are permitted in margarine *(6)*.

Although the experimental data cited above indicate that the hydrolysis products of isopropyl citrate and stearyl citrate esters, as well as mono- and diglycerides, are in most cases natural products and are non-toxic at relatively high doses, it is still necessary to prove that the unhydrolyzed compounds do not possess specific toxicities not revealed in studies of the individual components. For this reason, the experiments reported here on rats, rabbits, and dogs were carried out. No previous toxicological investigations have been reported on isopropyl or stearyl citrate esters.

The test compounds used are mixtures of mono-, di-, and tri-alkyl esters of citric acid. In Table 1 are listed the typical compositions of the compounds investigated. From the acid value and saponification value, it is possible to estimate the ratio of mono- to di- to tri-ester for each of the types under study. It will be noted that, in the case of isopropyl citrates, the mixture is predominantly mono-isopropyl citrate and that in the case of the stearyl citrates the mixture is predominantly distearyl citrate.

In the preparation furnishing the isopropyl citrate esters, there is about 62% of the mixture of mono- and diglycerides. Analyses conducted in our laboratory for the alcohol moieties in the isopropyl and stearyl citrate esters checked very closely with the calculated values confirming the composition of the esters as given in Table 1. Analyses for citric acid gave values somewhat below the calculated values.

It will be noted in the footnotes to Table 1 that there are some variations in the ratio of mono- to di- to tri-ester in each category. It is pertinent to point out that in the present toxicological studies and in the metabolic studies reported elsewhere *(3)* many different batches of the mixed esters were employed (12 different batches in the case of the isopropyl citrate preparation and 11 in the case of the stearyl citrates). Analysis of the individual batches indicated that all samples fell in the range of composition indicated in the footnotes to Table 1.

For the purpose of convenience in referring hereinafter to the test materials employed in this study, the isopropyl citrate esters, predominantly mono-isopropyl citrate will be referred to as IC. Similarly, the stearyl citrate esters, predominantly distearyl citrate, will be referred to as SC. When the isopropyl citrate ester, predominantly mono-isopropyl citrate, is fed in the mono- and diglyceride vehicle, it will be referred to as IC plus Vehicle.

TABLE 1

Typical Compositions of Compounds Investigated

Substance	Melting point, °C.	Acid value	Saponification value	Composition	Typical analyses [c]		
					Isopropyl alcohol [d]	Citric acid [e]	Stearyl alcohol [f]
					%	%	%
Isopropyl citrates in a vehicle consisting of mono- and diglycerides (1:1) of vegetable oil.	Liquid	160	370	27% mono-isopropyl citrate [g]	10.9	26.6	.....
				9% di-isopropyl citrate	(11.0)	(30.0)	.....
				2% tri-isopropyl citrate			
				62% mono- and diglycerides			
Stearyl citrates	57.8	88.6	248.1	12.5% mono-stearyl citrate [h]	.....	25.5	77.9
				75% distearyl citrate	.....	(28.3)	(76.8)
				12.5% tristearyl citrate			

[a] Figures in parentheses are calculated values.
[d] Isopropyl alcohol was determined after oxidation to acetone by refluxing with aqueous potassium dichromate and by precipitation with Deniges reagent *(40)*.
[e] Citric acid was determined on the aqueous extract after saponification by the method of Pucher, Wakeman, and Vickery *(28)* as modified by Perlman, Lardy, and Johnson *(27)*.
[f] Stearyl alcohol was determined as NSF by purification of the residue after saponification by filtration, drying, re-solution, and removal of the solvent.
[g] The 38% of isopropyl citrates is composed of about 65-80% mono-isopropyl citrates, 15-30% of di-isopropyl citrates and 5-10% tri-isopropyl citrate. Typical acid and saponification values are 410 and 690 respectively.
[h] The stearyl citrates are composed of about 10-15% monostearyl citrate, 70-80% distearyl citrates, and 10-15% tristearyl citrates.

The most important factors which determine the behavior of a substance in the body are its digestibility and absorption. If a substance is non-absorbable and at the same time non-irritating to the gastrointestinal tract, there is little possibility that it will exhibit any toxic effects. Data on the absorption and digestibility of isopropyl citrates, stearyl alcohol, and stearyl citrates have been presented in another communication *(3)*.

It was found that IC plus Vehicle in large amounts did not lower the high digestibility of margarine fat when fed to the rat or dog. On the other hand, when SC was fed at very high levels to the rat, the digestibility of the margarine fat was somewhat depressed. However, when the SC was given at a level of 3 times the maximal proposed level *(25)*, assuming that all the fat in the diet was so treated, the digestibility of the margarine fat was not appreciably altered *(3)*. In the case of the dog, no significant depression in digestibility of the margarine fat occurred when the level of SC in the fat was fully 80 times the maximal proposed level.

EXPERIMENTAL

In order to check for any possible toxicological effects of isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus the mono- and diglyceride vehicle (IC plus Vehicle), and of stearyl citrate esters, predominantly distearyl citrate, (SC), tests have been carried out on rats, rabbits, and dogs. The use of the several species in such studies is in line with the suggestions of Lehman, Lang, Woodard, Draize, Fitzhugh, and Nelson *(17)* that at least 2 species (including one which is a non-rodent) be employed.

All the rats used in the several types of experiments were from our stock colony. LD_{50} studies were conducted on IC, IC plus Vehicle, and on SC.

Acute tests were made on male and female rats at 3 levels for each test substance for a 6-week period. Control experiments in which no test substance was included in the diets were carried out in each series. In most cases, 10 rats of each sex were used per group.

The third type of test (chronic) employed with the rats was essentially a longevity experiment carried out over a period of 2 years with the same distribution in groups as in the acute tests. In both the acute and the chronic tests, the animals were sacrificed at the end of the experiment, and tissues were obtained for histopathological examination.

Another procedure used here for the dectection of any possible chronic or cumulative toxicity includes multigeneration studies. These involved raising the first generation of rats from weaning on the several test diets, and on a control diet, and continuing them on these regimens during the breeding and the lactation periods. When the young were weaned, the parents were discarded and the second generation was continued on the same diets which the parents had received. A similar routine was employed for succeeding generations through the weaning of the sixth generation, in the IC plus Vehicle tests, and of the fifth generation in the tests with SC.

In still another approach, additional groups of rats were used to ascertain whether these esters of citric acid in oils develop a toxicity when heated at 205°C. (a temperature used in deep frying) over a prolonged period (8 hrs.). The oils previously heated with or without the citric acid ester, as well as potato chips prepared by frying in such oils, were incorporated in diets which were then tested by growth studies on weanling rats over 11-week periods.

In the complete series of tests reported here (not including the absorption and digestibility studies), a total of 2398 rats was used.

Rabbits and dogs were employed for acute toxicity studies on the citric acid esters. The rabbit tests were continued for 6 weeks, while the experiments on dogs lasted for a 12-week period. A histopathological examination was made of the tissues of all of the test animals at the conclusion of the experimental period. In addition, massive single doses of IC plus Vehicle or of SC were administered to dogs. A total of 57 rabbits and 24 dogs was used in the toxicity tests.

The LD_{50} tests were carried out by the administration of the test substance by stomach tube to the fasted animals. In the rat tests, studies were made on IC, an equivalent isopropanol-citric acid mixture as control, IC plus Vehicle alone and in cottonseed oil, cottonseed oil alone as a control, and SC in cottonseed oil. The tests were considered positive if the animals died within periods of 4 to 6 days; this interval was chosen since, at that time, practically all the animals remaining alive were again gaining weight and in good nutritional condition. The dog experiments were carried out in essentially the same manner but because of necessity the number of test animals was restricted.

The diets used in the rat and dog experiments were made up of purified foodstuffs. The compositions of the various diets used are listed in Tables 2 and 3.

The rats used on the LD_{50} tests had been maintained on our usual stock diet. They were fasted for 18 hours prior to the administration of the test substance by stomach tube.

Rabbits were fed throughout the tests on commercial rabbit pellets.[9] IC plus Vehicle was mixed with the rabbit pellets in amounts of 0, 1.9, 3.8, 7.5, or 15.0% (0.0, 0.72, 1.4, 2.8, or 5.7% IC, respectively). In the tests with SC, the pellets were mixed with 2 or with 11% of the melted substance. The diets fed the dogs in the 3-month acute tests are patterned after those described by Cowgill *(4)*. Table 3 gives the composition of these diets.

The IC, IC plus Vehicle, and SC employed in the tests reported here were commercial products supplied by The Best Foods, Inc. The identity of the products was confirmed by our analysis. Hemoglobin determinations were made on the dogs by the acid hematin method.

[9]Albers Rabbit Family Ration.

TABLE 2

The Composition of Diets Used in the Rat Tests

Dietary component	Acute tests					Chronic tests				Multigeneration tests				Heated oil tests			
	70	80	81	82	83	84	85	86	71	72	87	88	73	89	74	90
Isopropyl citrate esters, predominantly mono-isopropyl plus mono- and diglyceride vehicle (IC plus Vehicle)																
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.9	54.3	53.0	50.5	55.5	55.5	55.5	50.5	53.5	50.5	53.5	55.5	55.5	44.0	44.0
Whole margarine[1]	12.50	11.42	10.34	8.00	3.50	12.22	11.94	9.70	12.5	12.5	9.7	9.7	....	....	....	....
Margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	11.5[j]	11.44[j]	....	....
Fat-soluble vitamin mixture[k]	....	....	....	....	....	....	....	....	....	....	....	....	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Liver powder[n]	....	....	....	....	....	....	....	....	5.0[o]	2.0[p]	5.0[o]	2.0[p]	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing IC plus Vehicle (0.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
IC plus Vehicle	....	1.68	3.36	7.00	14.00	0.28	0.58	2.80	....	....	2.8	2.8	....	0.06	....	....
IC content	....	0.64	1.3	2.7	5.3	0.11	0.21	1.1	....	....	1.1	1.1	....	0.02	....	....
Stearyl citrate, predominantly distearyl citrate (SC)																
Dietary component	170	180	181	182	183	184	185	186	171		187	188	73	189	74	190
	%	%	%	%	%	%	%	%	%		%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	19.0		19.0	19.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.2	53.0	50.5	45.5	55.0	53.5	45.5	51.8		51.0	43.3	55.5	55.5	44.0	44.0
Margarine fat	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.0		9.9	10.0	11.5[j]	10.64	....	....
Fat-soluble vitamin mixture[k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	7.6		7.6	7.6	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.8		4.8	4.8	4.0	4.0	4.0	4.0
Liver powder[n,o]	....	....	....	....	....	....	....	....	4.8		4.8	4.8	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing SC (7.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
SC	....	1.3	2.5	5.0	10.00	0.5	2.0	10.0	....		1.9	9.5	....	0.86	....	....

[1] 8 I U of vitamin D (as viosterol) added per g. food. [j] Heated for 8 hours at 205°C. [k] Composition was as follows: α-tocopherol, 0.5 g.; carotene (General Biochemicals, Inc.), 20 mg.; vitamin D_3 (crystalline Winthrop), 0.5 mg.; cottonseed oil to make a total weight of 100 g. [l] Anheuser-Busch, Strain G. [m] Osborne-Mendel salt mixture *(26)*. [n] Wilson Laboratories. [o] 1:20 concentrate. [p] 1:50 concentrate.

TABLE 3

Composition of the Diets Used in the Experiments on Dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine oil[r]	18.3	18.24	15.3
Salt mixture (Reference 41)	2.0	2.0	2.0
Cellu flour[s]	2.5	2.5	2.5
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Dried brewers' yeast[t]	5.0	5.0	5.0
IC plus Vehicle	....	0.06	....
SC	....	....	3.0

[r] Without additives. [s] Chicago Dietetics Supply House. [t] Anheuser-Busch (Strain G).

RESULTS AND DISCUSSION

LD_{50} Studies

The acute toxicity, as revealed by the determination of the LD_{50} levels, has been ascertained for rats, and checked with dogs. The results of the tests on rats are summarized in Table 4.

The LD_{50} values of the several test materials as determined on rats are all quite high. The LD_{50} value with male rats for IC plus Vehicle, when given alone, exceeded 20.7 g./kg., while when it was administered in a ratio of one part of cottonseed oil to 2 parts of IC plus Vehicle, the value was greater than 17.2 g./kg. The corresponding figures for the female rats were 18.8 and 19.2 g./kg. This would indicate that supplementation of IC plus Vehicle with fat does not accentuate the LD_{50} figure.

When IC was administered in alcohol solution or in water, the toxicity was 2.8 to 3.7 g./kg. The slightly lower LD_{50} value for the females receiving IC in ethanol may be the reflection of the higher alcohol dose which was administered. In the tests with females where an aqueous solution was employed, the figure for LD_{50} approximated the value obtained for the males. The quantity of IC required to produce 50% mortality approximated that for its hydrolysis products. However, when IC is administered in a fat vehicle, more than double the amount of IC is required to produce a 50% mortality.

The LD_{50} level for SC exceeds 5.4 g./kg. in the case of both male and female rats. Since it was impossible to prepare a solution of SC, liquid at body temperature, in a higher concentration than 20%, the maximum dose (30 ml./kg.) which could be introduced into the stomachs of rats contained only 5.4 g./kg.

Twelve dogs were used to check the LD_{50} values obtained with rats. Massive single doses, viz. 12 g./kg. of IC plus Vehicle, 2.25 g./kg. of IC, and 5 g./kg. of SC, were not fatal to the dog. After a period of 2 days, the dogs were eating normally and showed no obvious ill effects from the dosage.

TABLE 4

LD_{50} Tests on Rats

Substance tested	Sex of rats	Data on different levels of tests						Estimated LD_{50}[a]
IC plus Vehicle								g./kg.
Dosage (g./kg.)	M	2.1-20.7	.....	.....	.....	.....	.....	>20.7
Number of rats		6	.....	.....	.....	.....	.....	(>7.9)
Mortality (%)		0	.....	.....	.....	.....	.....	.....
Dosage (g./kg.)	F	2.7-15.5	17.7	21.0	23.6	27.0	.....	18.8
Number of rats		4	10	10	11	10	.....	(7.2)
Mortality (%)		0	30	90	89	100	.....	.....
IC plus Vehicle in cottonseed oil (2:1)								
Dosage (g./kg.)	M	0	11.1	14.1	16.1	17.2	.....	>17.2
Number of rats		10	9	10	9	9	.....	(>6.5)
Mortality (%)		0	11	20	22	33	.....	.....
Dosage (g./kg.)	F	0	12.0-13.4	15.8	17.9	19.2	22.2	19.2
Number of rats		10	20	10	10	10	10	(7.3)
Mortality (%)		0	0	20	10	50	50	.....
IC								
75% in 10% ethanol								
Dosage (g./kg.)	M	2.1	2.7	3.3	3.9	.....	.....	3.7
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		0	20	10	70	.....	.....	.....
15% in 10% ethanol								
Dosage (g./kg.)	F	2.1	2.7	3.3	3.9	.....	.....	2.8
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		10	40	90	100	.....	.....	.....
75% in water								
Dosage (g./kg.)	F	2.25	3.0	3.75	4.5	5.25	5.25[b]	3.6
Number of rats		10	10	10	10	10	10	.....
Mortality (%)		0	10	60	70	100	90	.....
Isopropyl alcohol plus citric acid in ethanol								
Dosage (g./kg.)	F	3.9[b]	3.9	5.25[b]	5.25[b]	.....	.....	4.5
Number of rats		10	9	10	10	.....	.....	.....
Mortality (%)		0	22	90	100	.....	.....	.....
SC (20% in cottonseed oil)								
Dosage (g./kg.)	M	0	0.9-1.8	2.7	3.6	4.5	5.4	>5.4
Sol. vol. (ml./kg.)		30	5-10	15	20	25	30	.....
Number of rats		10	4	2	12	13	10	.....
Mortality (%)		0	0	0	8	15	10	.....
Number of rats	F	10	4	2	12	13	10	>5.4
Mortality (%)		0	0	0	0	0	0	.....

[a] Figures in parentheses represent IC content of mixtures. [b] Male rats. [c] In water.

Short-Term (Acute) Tests

Experiments on Rats. Tests were made on IC plus Vehicle or SC when incorporated in the commercial margarine fat. Table 5 summarizes these data. No animals maintained on any of the diets died during the course of the tests.

There is no evidence, on the basis of the acute tests on adult rats, that either IC plus Vehicle or SC possesses any degree of toxicity. Thus, adult

TABLE 5

The Change in the Weight of Adult Rats Receiving Control Diets Alone or Similar Diets to Which Several Levels of IC plus Vehicle or SC Were Added

Diet no.	Test material in diet	Experiments on male rats						Experiments on female rats					
		Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten	Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten
			Start	End					Start	End			
	Isopropyl citrate esters, predominantly mono-isopropyl citrate, plus mono- and diglyceride vehicle (IC plus Vehicle)												
	%		g.	g.	g.	g.	g.		g.	g.	g.	g.	g.
70	0.0	10	336	386	50.2±2.7	625	0	10	221	247	26.5±3.5	496	0
80	1.68(0.64)[x]	10	326	364	38.3±5.3	568	9.54	10	208	230	21.8±4.6	447	7.51
81	3.36(1.8)	10	324	368	44.8±4.9	588	19.8	9	214	231	17.3±5.1	450	15.1
82	7.00(2.7)	9	331	373	42.0±6.7	597	41.8	10	210	226	15.5±3.5	437	30.6
83	14.00(5.3)	10	329	372	42.6±4.1	615	86.1	10	208	223	15.0±7.8	432	60.5
	Stearyl citrate esters, predominantly distearyl citrate (SC)												
170	0	10	203	271	67.9±5.9	442	0	10	202	230	27.6±3.4	390	0
180	1.3	10	206	302	96.0±7.9	514	6.68	10	202	230	28.4±3.6	422	5.48
181	2.5	10	209	292	82.7±7.5	494	12.3	10	206	235	29.2±3.8	406	10.1
182	5.0	10	205	296	90.8±7.8	542	27.1	10	196	220	25.0±5.5	447	22.3
183	10.0	10	208	304	95.4±6.9	555	55.5	10	193	220	27.2±5.2	445	44.5

[x] IC content of IC plus Vehicle in parentheses.

male rats receiving IC plus Vehicle at the highest level ingested 86 g. of this ester over a 6-week period (more than 2 g. per rat per day), and female rats ingested an average of 60 g. over the same period (1.42 g. per rat per day) without any demonstrable harmful effects. These correspond to a daily intake of 0.78 g. and 0.54 g. respectively of IC per rat per day in the 2 sexes.

The results of the acute tests on SC are quite similar to those on IC plus Vehicle. The average level of SC taken in by the male rats receiving the highest dosage was 55.5 g. (1.32 g. per rat per day) and that ingested by the females was 44.5 g. (1.06 g. per rat per day).

The slight average differences noted in the increases in body weight between the control animals and those receiving progressively increasing doses of IC plus Vehicle are not significant. These tests were conducted on adult rats of about 6 months of age and the growth responses show no parallelism with dosage. Likewise, no significance is attached to the apparently better growth responses of the adult animals receiving SC when compared to the responses of the control rats.

Experiments on Rabbits. Two types of experiments were carried out on rabbits receiving the IC plus Vehicle. In the first series of tests, the animals were fed on the basal diet (rabbit pellets) and the test substance was administered daily to the animals by stomach tube over the 6-week period. In the second and third series of experiments with IC plus Vehicle, and in all the tests with SC, the substances under examination were mixed with the crushed rabbit pellets before this material was fed to the rabbits. Table 6 shows the average results of these tests.

TABLE 6

The Change in Weight of Rabbits Receiving Control Diets or Those Containing Several Levels of IC plus Vehicle by Stomach Tube (Series I), or IC plus Vehicle or SC Mixed with Diet (Series II and III)

Group No.	Series	Test material in diet	Number	Body weight			Average gain in weight	Average total food eaten	Average test material given
				Start	3 weeks	6 weeks			
IC plus Vehicle by stomach tube									
		%		g.	g.	g.	g.	g.	g.
1	I	0	2	2010	2204	2770	760	Ad libitum	0
2	I	2.2	3	2098	2407	2899	801	food intake	120
3	I	4.4	2	2300	2598	2885	585	not record-	240
4	I	9.2	1	2104	2380	2780	676	ed.	500
IC plus Vehicle incorporated in diet									
5	III[7]	0	8	2503	2821	3263	760	5413	0
6	II	1.9	1	2092	2402	2735	641	5411	113
7	II	3.8	2	2026	2456	2985	959	5399	205
8	II	7.5	2	2550	2632	2902	352	5010	376
9	II	15.0	5	2166	2556	2927	761	5366	805
10	III[7]	11.3	8	2427	2952	3276	849	5175	585
11	III	22.5	8	2442	2702	3039	597	3803	856
SC incorporated in diet									
12	III	2	8	2532	2729	3171	640	4763	95.3
13	III	10	8	2516	2599	2950	434	4428	442.8

[7] One rabbit died during course of test.

Only 2 deaths occurred among the 57 rabbits during the course of the test. One of these was in Group 5 (control) and the second was in Group 10 (receiving 11.3% of the composite, IC plus Vehicle). The average intake of IC plus Vehicle of 4 groups of rabbits (22 animals) exceeded 500 g. during the 6-week period. The maximum consumption for the period was by one rabbit in Group 11. The total amounted to 1045 g., or 24.9 g. daily; this approximates a daily intake of IC plus Vehicle of 1% of the body weight.

The intake of the SC was somewhat lower, averaging 443 g. for the group on the higher dosage. The highest consumption in this group was 513 g. of the test substance for the period, or 12.2 g. per rabbit per day.

The average gain in weight for the control rabbits was 760 g. for the 6-week period. Several groups receiving IC plus Vehicle (Groups 2, 7, 9, 10) equalled or exceeded this figure. In both groups receiving SC, the average gain in weight was somewhat less. All rabbits receiving SC showed excellent increase in weight, except one animal which lost weight during the early part of the test when it failed to eat; this rabbit began to regain its weight during the last week of the test. It had returned to approximately its starting weight at the termination of the experiment.

A histopathological examination was made on 311 sections of 284 organs from 24 rabbits by one of us (H.R.F.). These included sections of the liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle of the 8 rabbits receiving the highest dose of IC plus Vehicle (22.5%), of the 8 animals given higher dose of SC (10%), and of the 8 controls (Group 5) in Series III. These were all males. There was no obvious morphological evidence of disease in the tissues of any group given diets in the categories listed.

Experiments on Dogs. Acute tests were made on 12 dogs over 11 to 12-week periods. Three groups of 4 each were used. The dietary regimens included a basal diet (control group), a similar diet including 0.06% IC plus Vehicle, and a third to which 3.0% of SC was added. The dogs used consisted of a litter of 6 cocker puppies, which were distributed equally between the groups, and 6 adult mongrels of unknown nutritional history. The adults were devocalized and dewormed before the test was started, while the puppies were devocalized during the second week of the tests. Because of the uniformity in origin and dietary background of the puppies, greater significance should be attached to weight changes of these animals.

All dogs received measured amounts of food once daily. In the case of the adult dogs, the amounts were first based on the quantities necessary to establish an optimum body weight, as calculated from Cowgill's formula *(5)*. When this weight was attained, they were placed on a maintenance diet. In the case of the puppies, the diet was adjusted to give a steady rate of growth. Water was given ad libitum throughout. The data on these tests are included in Table 7.

There is no evidence of toxicity of IC plus Vehicle or SC when fed to dogs over a 12-week period. IC plus Vehicle was fed at a level of 0.25% of the fat, which is 10 times the level proposed for its use in margarine (0.02% of the whole margarine or 0.025% of the margarine fat). SC was fed at a level of 14.8% of the fat, which is 80 times the maximum level to

TABLE 7

The Change in Weight of Dogs Receiving a Control Diet or Diets Containing IC plus Vehicle or SC

Dog No.	Type of dog	Sex	Body weight				Gain in weight	Total food eaten	Total test substance eaten	Hemoglobin at end of test
			Start	After 4 weeks	After 8 weeks	After 12 weeks				
			Control group (Diet D-1)							
			kg.	*kg.*	*kg.*	*kg.*	*kg.*	*g.*	*g.*	%
1	Cocker pup	M	2.3	3.2	3.5	3.6	1.3	7990	.....	12.9
2	Cocker pup	F	2.0	2.8	3.2	3.2	1.2	7927	.....	14.2
3	Mongrel adult	M	15.9	18.2	18.5	18.8[a]	2.9	27230	.....	15.9
4	Mongrel adult	M	13.5	15.4	15.9	16.3	2.8	16870	.....	17.6
Average[aa]			2.15	3.00	3.35	3.40	1.25	7958	.....	.....
			Group receiving IC plus Vehicle (Diet D-2)							
5	Cocker pup	F	2.3	3.3	3.7	3.8	1.5	7918	4.75	12.6
6	Cocker pup	F	2.5	3.1	3.6	3.8	1.3	7663	4.60	14.0
7	Mongrel adult	M	10.9	12.8	14.8	15.0[a]	4.1	23330	13.98	14.3
8	Mongrel adult	F	5.0	6.4	6.8	7.4	2.4	17290	10.37	18.6
Average[aa]			2.40	3.20	3.65	3.80	1.40	7790	4.68	.....
			Group receiving SC (Diet D-3)							
9	Cocker pup	F	2.1	3.2	3.7	3.8	1.7	7683	230.5	12.9
10	Cocker pup	F	1.8	2.6	3.2	3.3	1.5	7887	236.6	12.8
11	Cocker adult	M	12.7	15.5	17.1	17.3[a]	4.6	19320	579.6	13.6
12	Mongrel adult	M	5.2	4.8	5.0	5.7	0.5	7990	239.7	14.0
Average[aa]			1.95	2.90	3.45	3.55	1.60	7785	233.6	.....

[a] Weight at 11 weeks. [aa] Average of litter mate cocker puppies only.

be used in margarine (0.15% of the whole margarine or 0.19% of margarine fat).

The amount of food consumed in the 3 groups was comparable. Actually, in the case of the puppies, the gain in weight of the experimental groups somewhat exceeded that of the control group, although the former animals consumed somewhat less food. The same pattern was followed by the adult dogs. Hemoglobin levels determined before the dogs were sacrificed were practically identical for the several groups. A histopathological examination of the livers and kidneys of the dogs failed to reveal any evidence of pathological manifestations attributable to the test substance. Whatever divergencies from normal occurred were distributed equally among the several groups.

Long-Term (Chronic) Tests on Rats

Longevity Tests. In order to determine whether the materials tested may exhibit a toxicological action when their administration is continued over a prolonged period, chronic tests were carried out on rats for 2 years. The tests were discontinued at the end of this interval, so that the tissues could be obtained for pathological examination from animals which had remained alive until the termination of the experiment. Data on the body weights of the rats in the various series of tests at representative periods during the experiment, as well as on their survival, are included in Table 8.

With the exception of the control group in the distearyl citrate tests, 55% or more of the rats were still alive after receiving the various diets for a period of 2 years. When allowances are made for accidental deaths, the percentage survival in the tests with IC plus Vehicle were 59, 82, and 58% for the groups receiving the low, intermediate, or high level of test substance, with the value of 63% for the control group. In the experiments in which SC was added to the diets, the survivals amounted to 66, 65, and 55% for the groups receiving the progressively increasing proportions of the test compound, with an average of 44% for the corresponding control group.

No marked variations in weight between the experimental and the control groups were noted during the 2-year period. However, after 104 weeks, the average of the means of the weights of males and females still surviving in the IC plus Vehicle tests were as follows: Diet 70 (control), 414 g.; Diet 84 (0.28%), 400 g.; Diet 85 (0.56%), 425 g.; and Diet 86 (2.8%), 404 g. In the tests with SC, the corresponding average weights were: Diet 170 (control), 380 g.; Diet 184 (0.5%), 486 g.; Diet 185 (2.0%), 413 g.; and Diet 186 (10%), 388 g.

Histopathological sections of liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle or ovary were examined. In the case of the rats receiving IC plus Vehicle at a 2.8% level, it is reported as follows: "It is my opinion that no specific pathological lesions developed which could be attributed to the product fed."[bb] An examination of the same tissues of a series of rats which had

[bb] Report of Professor Ernest M. Hall, Department of Pathology, School of Medicine, University of Southern California.

TABLE 8

The Body Weights and Survivals of Rats Receiving Control Diets or Diets Containing Several Levels of IC plus Vehicle or SC

Category	IC plus Vehicle tests				SC tests			
	Diet 70	Diet 84	Diet 85	Diet 86	Diet 170	Diet 184	Diet 185	Diet 186
Test substance in diet, %	0	0.28	0.56	2.80	0	0.5	2.0	10.0
IC content	0	0.11	0.21	1.06	....	....	....	....
Male rats								
Number in group	10	10	10	10	10	9	10	10
Body weight:								
Start, g.	35	36	36	36	35	33	34	32
After 32 weeks, g.	339	362	346	324	361	361	338	328
After 64 weeks, g.	421	471	452	419	423	475	423	423
After 96 weeks, g.	488	546	507	467	471	597	488	474
After 104 weeks, g.	520	495	543	461	454	610	524	482
Rats alive at end	7	5	5	4	3	4	6	5
Female rats								
Number in group	10	10	10	10	10	10	10	10
Body weight:								
Start, g.	40	38	39	39	30	32	32	28
After 32 weeks, g.	228	230	216	222	220	222	208	217
After 64 weeks, g.	287	268	263	255	267	282	272	259
After 96 weeks, g.	308	296	316	328	297	359	294	309
After 104 weeks, g.	309	304	307	348	305	363	302	294
Rats alive at end	5	5	9	7	5	8	7	6
Survival of all rats								
Uncorrected, %	60	50	70	55	40	60	65	55
Corrected for those killed accidentally, %	63	59	82	58	44	66	65	55

received distearyl citrate at a 10% level for 2 years was made by one of us (H.R.F.). Although most of the rats exhibited some diseased tissues characteristic of old age, no specific changes traceable to the diet could be noted. Metastatic calcification and tumor formation were noted in certain tissues examined; however, they are not considered to have special significance, inasmuch as they were noted in the tissues of both test and control rats.

Multigeneration Tests. The most sensitive method for the detection of dietary deficiencies or toxicity involves subjecting several generations of the animals to the test diets. All the factors of stress are brought to bear on the animal in such tests, namely growth, pregnancy, and lactation. Diets which are entirely satisfactory for maintenance, or even for growth, over a single generation may fail to be sufficient for reproduction; moreover, a dietary regimen which serves adequately for both growth and reproduction may be unsatisfactory for lactation. The latter stress offers the most severe test of the nutritional value of a diet.

In the multigeneration tests, weanling male and female rats (Generation 1) whose parents had been on the laboratory diet were raised on the test diets and were bred after 10 weeks (at 13 weeks of age). These rats were continued on the test diets during the subsequent pregnancy and lactation. Young from the first generation (Generation 2) were continued after weaning on the same test diets which their parents had received. At maturity the second-generation rats were bred and a routine similar to the above was repeated. In the case of the rats receiving IC plus Vehicle, the experiment was continued through the weaning of the sixth generation; in the tests with SC, the experiment was terminated when the fifth-generation rats were weaned. Table 9 summarizes the data on the growth of the successive generations, while Table 10 records the information to be gained from the pregnancy and lactation performances.

The multigeneration tests indicate that IC plus Vehicle is completely harmless when incorporated in the diet at a level of 2.8% (equivalent to 1.1% of IC). The rats remained fertile throughout the 5 generations. The total litters cast in the test group comprised 91% of the females bred (59 of 65); this figure was identical with that of the control group (90%, 65 of 72). The body weights of the progeny were likewise similar in the control and experimental groups. At the age of 3 days, the average weights were 7.6 and 7.5 g., while at the age of 21 days the mean values were found to be 34.5 and 35.1 g., respectively.

The results of the growth tests carried on from weaning for the successive 10-week period give added confirmation of the non-toxicity of IC plus Vehicle at the 2.8% level. The average 10-week weight of the fifth generation male and female rats on the test diet (254.7 g., 178.3 g.) not only exceeded the average of the first-generation rats on this test diet for the 10-week period (238.7 g., 176.6 g.) but it was also higher than that of the fifth generation of the control rats (223.4 g., 151.2 g.).

A similar analysis of the data indicates that SC exhibits no toxicity over 4 generations when fed at a level of 1.9% or 9.5% of the diet. The fertility remained high in all groups. The percentages of successful pregnancies were as follows: Diet 171 (control), 93% (55 of 59); Diet 187

TABLE 9

Body Weights of Successive Generations of Rats Receiving Control Diets or Those Containing IC plus Vehicle or SC

Diet No.	Test substance in diet	Generation	Male rats				Female rats			
			Number	Body weights			Number	Body weights		
				Start	After 5 weeks	After 10 weeks		Start	After 5 weeks	After 10 weeks
IC plus Vehicle tests										
	%			g.	g.	g.		g.	g.	g.
71	0	1	10	30.1	158.6	246.9	15	30.5	123.6	175.3
		2	9	38.6	168.5	281.5	15	37.8	137.9	187.3
		3	10	36.5	151.1	243.0	16	34.5	113.2	157.1
		4	14	38.8	153.2	224.3	22	37.4	123.0	159.4
		5	13	37.5	143.8	223.4	26	35.9	113.8	151.2
87	2.8	1	11	30.4	155.2	238.7	15	30.3	133.0	176.6
		2	11	38.5	157.7	265.0	12	34.6	132.8	182.1
		3	17	35.0	134.7	227.5	16	34.5	121.2	174.7
		4	12	39.5	164.4	240.0	18	37.9	124.5	160.4
		5	17	38.7	178.1	254.7	21	37.6	136.3	178.3
SC tests										
171	0	1	5	37.8	164.0	265.0	10	37.3	135.7	182.5
		2	14	34.0	156.6	243.6	21	35.7	125.2	166.3
		3	16	37.4	168.2	253.5	19	35.8	122.7	163.1
		4	18	36.9	161.7	289.3	22	36.5	126.1	179.0
187	1.9	1	5	38.2	167.4	273.0	10	37.3	132.8	177.5
		2	19	34.9	150.1	245.3	16	33.9	122.0	171.1
		3	14	37.1	175.2	257.8	20	35.5	131.7	172.9
		4	10	40.3	172.9	268.4	16	38.6	129.1	177.8
188	9.5	1	4	36.5	160.5	267.3	10	37.6	131.5	177.4
		2	19	36.6	153.7	246.0	26	32.5	125.3	165.4
		3	10	33.2	162.2	237.1	19	32.7	124.7	166.2
		4	13	43.8	174.9	273.3	19	39.5	132.0	178.8

TABLE 10

Pregnancy and Lactation Response of Successive Generations of Rats Receiving Control Diets or Diets Containing IC plus Vehicle or SC

Diet No.	Test substance added	Parents				Litter					
		Generation	Number of rats bred	Number of litters cast	Average time[cc]	Generation	Average number	Body weight		Total rats weaned	Died between 3rd and 21st day
								3 days	21 days[dd]		
IC plus Vehicle tests											
	%				*days*			*g.*	*g.*		
71	0	1	15	13	31.0	2	8.2	7.6	38.3(49)	70	0
		2	10	10	43.2	3	6.2	7.3	28.1(14)	33	4
		3	10	10	35.4	4	6.9	8.0	38.6(28)	56	3
		4	22	17	34.9	5	6.4	7.7	37.0(21)	55	1
		5	25	25	36.1	6	5.8	7.3	30.4(28)	103+[ee]	1
87	2.8	1	12	12	28.8	2	9.9	7.5	35.8(77)	77	0
		2	8	8	31.4	3	8.3	6.9	34.5(35)	35	9
		3	7	7	38.2	4	6.7	7.7	35.2(14)	46	0
		4	17	17	29.6	5	7.9	8.2	34.3(91)	98	0
		5	21	15	42.2	6	6.3	7.2	35.9(28)	56+[ee]	0
SC tests											
171	0	1	10	9	28.0	2	9.4	6.9	34.0(35)	35	0
		2	14	13	27.3	3	8.3	6.6	34.3(35)	60	3
		3	19	17	27.7	4	7.7	7.2	33.6(35)	65	5
		4	16	16	26.6	5	7.1	6.9	34.5(63)	72	7
187	1.9	1	10	10	26.8	2	6.3	6.7	34.6(21)	41	0
		2	15	15	26.2	3	6.8	7.4	33.5(42)	58	0
		3	15	14	24.4	4	8.1	7.0	36.9(56)	83	3
		4	14	14	31.0	5	8.4	6.8	36.7(56)	71	2
188	9.5	1	11	11	30.2	2	7.6	7.0	34.5(43)	61	2
		2	23	23	26.2	3	8.5	7.2	35.5(105)	117	0
		3	16	14	26.5	4	8.5	8.5	41.0(56)	67	0
		4	14	14	27.4	5	9.4	7.6	37.1(84)	94	0

[cc] Period elapsing after the male and female rats were put in the same cages. [dd] Average weights of 7-litter rats only. Figures in parentheses give average number of rats. [ee] Experiment terminated before all rats were weaned.

(1.9% SC), 98% (53 of 54); and Diet 188 (9.5% SC), 97% (62 of 64). A 100% fertility was obtained in the fourth-generation rats from the control and both experimental groups. The body weights of the young were slightly higher in the experimental groups than in the control group; this would indicate that no interference with the lactation process had been occasioned by SC. The average weights of 3-day-old rats of all 4 generations were 6.9 g. for the control group, 7.0 g. for those receiving 1.9% SC, and 7.6 g. for those receiving 9.5% of SC in the diet. After 21 days, the comparable averages were 34.1, 35.4, and 37.0 g., respectively. Mortality from the 3rd to the 21st day was 15 for the control group, 5 for those which received 1.9%, and 2 for those which were fed 9.5% of SC.

In the 10-week growth tests, the averages of the mean weights of the 4 generations on the several dietary regimens showed little difference. The values for the male rats gave a grand average of 262.8, 261.1, and 255.9 g. for Diets 171, 187, and 188, respectively. In the case of the females, the corresponding grand averages were 172.7, 174.8, and 172.4 g., respectively. The mean weights of the rats after 10 weeks on both experimental diets containing SC were approximately identical, after 4 generations, with those of the first-generation animals on the same dietary regimen.

Growth Tests on Rats with Diets Containing Heated Fats Without or With Citric Acid Esters

Since it is known that some fats such as sardine oil become less completely digestible when subjected to a high temperature *(15)*, it was considered desirable to make tests on margarine fat heated without or with IC plus Vehicle or SC to determine whether a change in nutritional value may occur. In order to establish whether such a decrease occurs, growth tests were made on weanling rats using fats so treated as well as potato chips prepared by deep-fat frying in such fats. If the growth responses of the heated fats containing either of the citric acid esters tested were found to be inferior to that of the control heated fat, such an effect might be ascribed either to decomposition products of the citric acid esters or to a decomposition of the fat caused by heating in the presence of the citric acid esters. Data on these tests are summarized in Table 11.

No adverse effects on growth were noted in either male or female rats over 10-week periods when margarine fats heated at 205°C. continuously for 8 hours and used for deep-fat frying were incorporated in the diets, as compared with control tests in which unheated margarine fat was employed in similar dietary regimens. Moreover, when IC plus Vehicle or SC was added to the test oils prior to the prolonged heating procedure, and these oils were then subjected to deep-fat frying for the 8-hour period, no toxicity developed. This fact was demonstrated by identical growth curves of rats receiving diets containing the fat heated with additives as compared with those of rats receiving diets containing the heated fat without additives as well as the unheated fat without additives.

In addition, no differences in the growth of weanling rats were observed when they were fed diets containing potato chips fried in margarine fats with additives over that noted when the potato chips had been prepared in margarine fat without these citric acid esters. These results indicate

TABLE 11

The Increase in Weight of Weanling Rats over 10-Week Periods Which Received Diets Containing Unheated Margarine Fat (Control), Margarine Fat Heated at 205°C. for 8 Hours Without or With IC plus Vehicle or SC, and Potatoes Fried in Such Fats

Diet No.	Category	Test material in diet[ff]	Number of rats	Body weight		Gain in weight	Total food eaten	Total test substance eaten	Efficiency of diet[gg]
				Start	End				
	Experiments on male rats								
		%		g.	g.	g.	g.	g.	
70	Unheated fat (Control)	0	10	35.4	258.2	222.8	819	0	6.68
73	Heated fat	0	10	36.2	265.5	229.3	758	0	7.08
89	Heated fat plus IC plus Vehicle	0.06	10	37.3	262.3	225.0	748	0.45	7.04
74	Potato chips from 73	0	10	39.9	260.4	221.4	758	0	7.43
90	Potato chips from 89	0.044	9	36.3	263.0	226.4	789	0.35	7.04
189	Heated fat plus SC	0.86	10	38.0	267.5	229.5	776	6.67	6.93
190	Potato chips from 189	0.68	11	39.0	265.1	226.1	793	6.40	6.68
	Experiments on female rats								
70	Unheated fat (Control)	0	10	39.7	180.2	140.5	701	0	4.93
73	Heated fat	0	10	38.1	187.0	148.9	670	0	5.20
89	Heated fat plus IC plus Vehicle	0.06	9	38.0	193.7	155.7	680	0.41	5.37
74	Potato chips from 73	0	9	37.2	182.1	144.9	677	0	5.42
90	Potato chips from 89	0.044	9	39.2	185.7	146.5	689	0.31	5.22
189	Heated fat plus SC	0.86	9	38.9	180.8	141.9	649	5.58	5.12
190	Potato chips from 189	0.68	8	39.4	181.3	141.9	699	5.65	5.00

[ff] Based on content of citrate esters present in fat at start of heating. [gg] (G. gain in weight/calories consumed) × 100.

that the heating of IC plus Vehicle or SC in margarine oil for 8 hours at 205°C. does not produce toxic decomposition products nor does it cause the oil to be adversely affected.

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SUMMARY AND CONCLUSIONS

The results of the present toxicological study of 2 new chemicals proposed for use as food additives may serve as an illustration of what constitutes adequate testing of new food chemicals. Mixtures of mono-, di-, and tri-alkyl esters of citric acid were evaluated for the safety of their use in stabilizing the flavor of oils and fats and foods containing such oils and fats.

Consideration was first given to an independent analysis on the part of the testing laboratory of the mixed esters of citric acid to check the claims made by the manufacturer that the products were actually what they were reputed to be. Because of the variations in composition of mixed esters, many different batches were employed in the current investigation; analyses were made of representative batches.

Consideration was then given to the possible hydrolytic products of the esters of citric acid, and evidence was cited from the literature showing that such hydrolytic products in themselves exhibit no toxicity. It was emphasized that the intact esters themselves must be subjected to exhaustive toxicological studies using 3 species, not more than 2 being rodents. LD_{50} values carefully determined on one species and checked with another, acute experiments on 3 species, longevity tests, multigeneration studies, and toxicological studies of the possible degradation products of the citric acid esters were all conducted. In addition, histopathological examinations of tissues obtained from sacrificed animals were made throughout the course of the investigation. When the major portion of the studies was completed, the data were presented to the Division of Pharmacology of the U. S. Food and Drug Administration for their criticism, and their suggestions were followed in carrying the toxicological investigation to completion.

More specifically, studies have been undertaken to determine whether 2 new flavor-stabilizing agents for fat products, namely isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus mono- and diglycerides vehicle (IC plus Vehicle), and stearyl citrate esters, predominantly distearyl citrate (SC), possess any toxicity when measured by various appropriate indices.

The LD_{50} values for the 2 compounds studied were relatively high. That for IC plus Vehicle alone or dissolved in oil was approximately 19.0 g./kg. (7.2 g./kg. as IC), while the figure for SC exceeded 5.4 g./kg. in rats. No sex differences were noted.

There is no evidence from acute experiments on rats (6 weeks), rabbits (6 weeks), or dogs (11-12 weeks) of any toxicity as measured by growth, mortality, or pathological manifestations.

Longevity (chronic) tests were carried out on rats, for 2-year periods from weaning, on IC plus Vehicle at several levels (0.28, 0.56, and 2.80% of the diet) and on SC at 3 levels (0.5, 2.0, and 10.0% of the diet). No deleterious effects as compared with that of the controls were noted in any groups in so far as could be determined by growth rates, mortality, and histopathological examinations of tissues.

Another method for evaluation of possible chronic toxicity, namely multigeneration studies, likewise indicated that IC plus Vehicle, at a level of 2.8% of the diet, and SC, at levels of 1.9 and 9.5% of the diet, are completely innocuous. These conclusions are based upon fertility over 5 and 4 generations, lactation performance as judged by the 3-day and 21-day weights of the offspring, as well as by their survival for the nursing period, and by the rate of growth of rats of the several generations over a 10-week period following weaning.

Margarine fat containing 0.5% IC plus Vehicle or 7.5% of SC did not develop toxicity (as determined by rat feeding tests over 12 weeks) when subjected to deep-fat frying at 205°C. over an 8-hour period. Potato chips, fried in such fats, and which retained approximately 40% of the fat, were also entirely satisfactory for growth.

Histopathological examination of the tissues of the rats after 2 years, of rabbits after 6 weeks, and of dogs after 11 to 12 weeks on the test diets did not reveal any evidence of specific damage. In the case of rats and rabbits, the following tissues were subjected to histopathological examination: liver, kidney, gastrointestinal tract, spleen, heart, lung, pancreas, gonads, brain, and adrenals. Only the liver and kidneys were examined in the dog tests.

All tests for toxicity indicate that IC when given as IC plus Vehicle and SC are harmless at levels as high as 1.1 and 10% of the diet, respectively. The maximum level at which IC has been tested and proved to be harmless is more than 500 times that which would be ingested in margarine containing 0.02% of IC where this fat makes up 15% of the diet by weight. Similarly, the highest level at which SC has been tested and which has proved harmless is about 500 times greater than would be consumed when margarine containing 0.15% of SC makes up 15% of the diet by weight.

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BACTERIOLOGICAL ANALYSIS OF EDIBLE GELATIN[a]

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In order to prevent highly contaminated and inferior gelatin being used in food products, Canada in 1936 enacted regulations under the Food and Drugs Act specifying that edible gelatin should contain no more than 10,000 bacteria per gram (plate count), with coliform organisms absent from 0.01-g. portions. The effectiveness of this step is shown by the decline in the percentage of sub-standard samples from 43.1% in 1936 to 4.9% in 1939 and to 1.6% in 1946.

In carrying out the official analysis, an 11-g. portion is weighed into a 99-ml. dilution blank, and tempered in a 45°C. water bath for not over 20 minutes with frequent agitation to hasten solution; further dilutions are prepared and plated as directed *(1)*. Where a sample fails to meet these specifications, the analysis is repeated on a second 11-g. portion.

TABLE 1

Variations in Bacteria Count Between Original and Repeat Analyses of 11-g. Portions of Powdered Gelatin

Sample No.	Count per gram	
	Original	Repeat
659	12,000	<50
661	16,000	50
823	11,000	600
1024	26,000	600
1064	29,000	350
1777	7,600	45,000
1785	36,000	4,600
1982	19,000	100
1983	28,000	110,000
1992	5,500	120,000
2019	57,000	1,500

While the results from the repeat analysis generally confirm the original finding, a number which show high counts on the initial analysis fall within the limit on retesting. In some instances the results of the two analyses are sharply at variance. Table 1 shows some of the more striking divergencies found during the past six months. Faulty technique can scarcely be responsible as the same phenomenon has been encountered by eight different workers during the past 30 months.

The Technical Committee of the Edible Gelatin Manufacturers Research Society of America *(3, 4)* have reported on the basis of their

[a] Contribution No. 303 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

comparative studies that "variations in total count of 200 to 500% may be expected as normal" *(4)*. Their data show such variations between the results reported by six collaborating laboratories, where average counts on five replicate portions, plated in duplicate, were compared. Nevertheless, variations such as those shown in Table 1 of the present paper were great enough to warrant a search for the reason.

EXPERIMENTAL

Dry Gelatin. If, as we believe, the variations noted are due to the highly irregular manner in which bacterial cells and clumps are distributed in powdered gelatin, then these variations should increase as the size of sample analysed is reduced. Extensive experiments, of which the data in Table 2 are representative, have confirmed that this

TABLE 2

Variability of Replicate Analyses[b] *in Relation to Size of Sample*

Sample	A			B			C		
Size of portion analysed	11 g.	1 g.	0.1 g.	11 g.	1 g.	0.1 g.	11 g.	1 g.	0.1 g.
Mean count per g.	895	3,960	4,810	6,550	4,925	3,105	3,155	5,690	19,450
Minimum	300	450	350	3,100	1,500	450	850	950	400
Maximum	1,400	8,850	24,500	12,500	13,800	10,500	5,750	22,850	135,500
Ratio Min.: Max.	1:4.7	1:40	1:70	1:4	1:9	1:23	1:6.7	1:57	1:340

[b] Duplicate plates were poured for each of 10 replicate portions of each size of sample.

is indeed the case. Table 3 indicates how wide these variations are when numerous (30 to 40) 0.1-g. portions are analysed separately, while Table 4 summarizes results obtained when 10 replicate 11-g. portions were compared with 10 replicate 1-g. portions on four different occasions.

In an attempt to obtain a more uniform distribution of the bacteria in gelatin, selected samples were shaken vigorously on a horizontal reciprocating shaker for 1 hour. Others were mixed by pouring back and forth 10 times from one sterile container

TABLE 3

Variations in Counts from Replicate 0.1-g. Portions of Gelatin

Sample	No. of replicates	Minimum	Maximum	Average	Original count on 11-g. portion
A	40	<100	88,000	5,200	12,000
B	30	<100	6,700,000	540,000	250
C	40	<100	310,000	35,000	18,000
D	40	<100	2,900	260	<50
E	40	7,300,000	33,000,000	18,000,000	T.N.T.C.
F	40	50	1,200,000	68,000	1,000
G	40	<100	4,300	.....	14,000

to another. Although some improvement was effected, variations of over three-hundred-fold were still occasionally encountered between replicate 1-g. portions. Further attempts were made using a Waring blendor to mix the dry powder. Data from a typical experiment (Table 5) show that the bacterial content was not uniformly distributed even after two minutes treatment, while the average count was reduced.

TABLE 4

Variability of Replicate Analyses[e] in Relation to Size of Sample

	Sample			
	D	E	F	G
11-g. portions				
Mean count per g	40,530	1,360	105,414	7,224
Maximum	78,600	3,740	294,600	18,720
Minimum	22,420	280	30,760	1,600
Ratio min.: max	1:3.5	1:13	1:9.6	1:12
1-g. portions				
Mean count per g	1,920	146	4,704	4,016
Maximum	10,020	420	25,000	11,500
Minimum	100	20	20	100
Ratio min.: max	1:100	1:21	1:1,250	1:115

[e] Quintuplicate plates were poured for each of 10 replicate portions of both sizes of sample.

TABLE 5

Effect of Mixing Dry Powdered Gelatin in Waring Blendor (Plate counts[d] per gram from 1-g. portions)

Portion No.	Period of mixing in seconds						
	0	5	10	15	30	60	120
1	14,000	8,600	1,800	6,500	10,000	1,300	10,000
2	10,000	5,300	2,600	2,800	2,300	7,500	3,600
3	2,900	1,300	14,000	5,700	2,300	3,300	7,100
4	3,000	5,000	5,700	3,700	5,000	5,900	1,800
5	14,000	29,000	4,600	3,500	14,000	4,300	2,000
Mean	8,780	9,840	5,340	4,440	6,720	4,560	4,900
Minimum	2,900	1,300	1,800	2,800	2,300	1,800	1,800
Maximum	14,000	29,000	14,000	6,500	14,000	7,500	10,000
Ratio min.: max	1:4.8	1:23	1:7.7	1:2.4	1:6.1	1:4.2	1:5.5

[d] Averages of duplicate plates.

Dissolved Gelatin. Duplicate plates poured in routine control of edible gelatin frequently showed much wider variations[*] than are generally noted with other food products. This could be due to incomplete solution of the gelatin, or to incomplete disintegration of bacterial clumps. In the hope of overcoming this, a non-ionic wetting agent (0.1% Tween 80[f]) was added to the dilution water. This was subsequently repeated, allowing the primary dilution to stand at room temperature for 30 min. before tempering at 45°C.; these attempts were without noticeable effect.

Since February 1950, the routine primary (1/10) dilutions, after tempering at 45°C. for about 20 min., have been shaken for 10 min. on a wrist-action shaker to obtain more complete solution. The dilution bottle also contained broken glass as an additional aid. Even with these extra steps, some samples showed a few small undissolved particles under the microscope. The effect of an additional 10 min. of shaking was studied, but results were variable. In one case the variability between replicate plates was reduced, but the average count dropped sharply; in several others there was no consistent effect.

Up to this time the practice had been to add the powdered gelatin to the dilution water at room temperature, shake thoroughly, then place in the water bath to dissolve

[*] Of 201 consecutive samples, 7.4% showed differences between duplicate plates of 100 to 400% while 4.5% showed more than 400%.

[f] We are indebted to G. F. Sterne & Sons Ltd., Brantford, Ont., Canadian agents for Atlas Powder Co., Wilmington, Del., for a supply of Tween 80. The use of this product was suggested by Dean B. A. Eagles, University of British Columbia, Vancouver, B. C.

without delay. It was felt that the dissolving of poorly soluble samples might be facilitated by a preliminary soaking period to permit rehydration of the gelatin particles prior to tempering at 45°C. Such a step is already part of the procedure in the laboratory of one large manufacturer.[5] The value of prolonging the tempering period at 45°C. also warranted study. Consequently a series of tests was conducted, using 11-g. portions from a large sample of gelatin, which were treated as follows: A. After adding 99 ml. of dilution water at room temperature, the contents of the flask were mechanically shaken for 10 min. then placed at 5°C. for about 30 min. The flask was then placed in a 45°C. water-bath with frequent vigorous shaking until the gelatin appeared to be

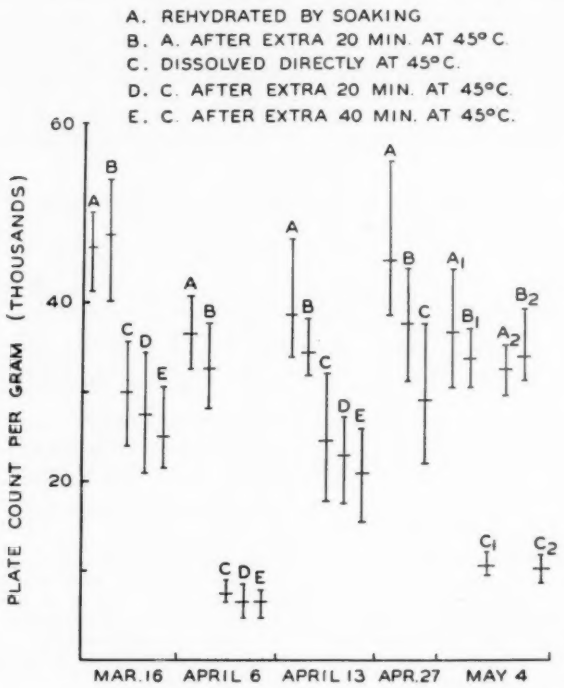

FIG. 1. Data from repeated replicate platings of 11-g. portions of one gelatin sample. Each vertical bar represents the spread between the highest and the lowest count from 10 replicate plates, with the arithmetic mean indicated by the middle cross-bar. The May 4th data represent parallel platings by two analysts from the same dilutions.

dissolved; it was then shaken mechanically for 10 min., a 1/100 dilution prepared, and 10 replicate plates poured. B. The primary dilution from A was returned to the water-bath for a further 20 min., then a 1/100 dilution prepared from which 10 replicate plates were poured. C. Eleven g. gelatin were added to a 99-ml. dilution blank, tempered in the water-bath at 45°C. for 20 min., then mechanically shaken for 10 min. before plating. D. The primary dilution from C was returned to the water-bath for 20 min., then a further set of 10 plates poured. E. The primary dilution from C was again returned to the water-bath for a further 20 min., then a final set of 10 plates poured.

[5] Thanks are extended to Mr. H. B. Richie, Research Laboratories, Swift and Co., Chicago, for a description of the technique employed there.

Five replications of this test were made on the same sample, the summarized results of which appear in Fig. 1. The data for May 4th represent platings from the same dilutions by two different workers. It would appear from this that soaking increased the count (compare A and C), while the variations between counts on replicate plates were slightly decreased. This sample showed much more uniform distribution of bacteria than many we have examined. What is of importance, however, is the much better agreement between the average counts for the soaked 11-g. portions (A) than for the unsoaked portions (C) on the five different trials. With the former, they varied between 32,100 and 46,100, while with the latter the values ranged from 7,200 to 29,600 per gram. Presumably the soaking softened bacterial clumps, and their subsequently greater disintegration resulted in higher and more uniform counts. Additional tempering at 45°C. nearly always led to a slight drop in count, but the differences were small. Although no check on pH was made on this sample, it is possible that the drop in count was due to a high hydrogen ion concentration, since other samples have shown no such reduction in count when held for up to 90 min. at 45°C. Since soaking has been adopted as a standard practice, there has been somewhat better agreement between replicate analyses and replicate plates. It has not however, overcome the basic problem posed by the highly irregular manner in which bacteria are distributed in powdered gelatin. (See Tables 2 to 5 and Figure 1.)

Optimum Temperature of Incubation. When bacteriological standards for edible gelatin were established in Canada in 1936, plates were required to be incubated at 28-30°C. for 72 hours. In 1943 this was changed to 37°C. for 48 hours. With the official adoption of lower incubation temperatures for milk analysis, comparisons have been made between 37° and 32°C. incubation for 48 hours. Of 186 samples from a variety of sources, 79 gave higher counts at 37°C., 97 gave higher counts at 32°C., and 14 were identical. On the other hand, previous comparisons *(3)* between 35-37°C. and 28-30°C. generally gave slightly higher counts at the higher temperature. In view of the high degree of variation inherent in this product, it is believed that these differences are not important and that either temperature may be used for this purpose.

Cultures for coliform organisms have always been incubated at 37°C. in this laboratory. Comparative tests with a small series of samples showed 16 to be positive in lactose broth at 37°C. but negative at 32°C., 7 positive at both temperatures and none positive at 32°C., and negative at 37°C. The higher temperature is obviously more suitable.

DISCUSSION

The data presented indicate that the distribution of bacteria in some samples of powdered gelatin is extremely irregular. This not only leads to disagreement between results of different laboratories, but also between tests within the same control laboratory. This same variability makes it difficult to assess the effect of any step in the analytical procedure, since an apparent drop in count may not be due to the factor under study, but solely to chance. Consequently, many conclusions have had to be modified as further data were obtained.

While prolonged mechanical shaking, or mixing in a Waring blendor was of some value in reducing the variations in count, variations were still far too wide to be regarded as satisfactory, and, with the Waring blendor, the count was reduced in every case. Furthermore, such time-consuming procedures are scarcely practicable where large numbers of samples must be analysed without delay.

From their studies *(3)* on the bacteriological examination of 30 to 40 mesh gelatins, the Technical Committee of the Edible Gelatin Manufacturers Research Society of America concluded it was preferable to prepare a 1% solution from a 1-g. portion. Reasons given for preferring a 1% to a 5% solution include greater ease of dissolving, fewer spreaders, and saving of time, glassware, etc. While admitting these advantages, our results lead us to question their conclusion that "as the accuracy and

reliability of the counts made on the one-per cent solutions are probably greater than those made on the five-per cent solutions, it would seem that the use of the one-per cent solution is preferable". Our results all point to the desirability of reducing the sampling error (resulting from the uneven distribution of bacteria) by examining as large a portion of gelatin as is practicable. In every instance where replicate 11-g. and 1-g. portions have been analysed, the counts from the 11-g. portion have shown much closer agreement than those from the 1-g. portions, despite the greater ease with which the latter portions can be dissolved.

It has been contended *(2)* that the preliminary soaking, together with the longer time required to dissolve the 11-g. portion, favors bacterial multiplication and hence does not reflect the true bacterial content of the gelatin. We have held a number of 1/10 and 1/100 dilutions prepared from 11-g. portions at room temperature and at 45°C.; there has been no evidence of multiplication after holding up to 4 hours at room temperature, or up to 90 minutes at 45°C. This has also held true for dried milks, according to tests carried out by Mr. A. H. White of this Division.

A possible explanation for the divergent views on this point may be that the samples examined by the Technical Committee of the Edible Gelatin Manufacturers Research Society of America represent the products of up-to-date plants where equipment and methods leave little to be desired. Products from such plants should contain few bacteria, and such marked variations as we have observed should rarely be encountered. Our laboratory, on the other hand, examines more diverse samples coming from plants in Australia, Britain, France, Belgium, and Holland as well as North America. It may be that the products of certain of these plants are more prone to show extreme variability than others. Examination of our records lends some support to the hypothesis, although a given plant may sometimes furnish a moderately high count product showing excellent agreement between replicate determinations, and at other times a product giving widely varying counts from the same batch or the same sample. Whatever the true explanation may be, it is felt that as large a sample as is practicable should be used in the official control of edible gelatin.

While the bacteriologist must always strive to obtain reliable counts, the basic problem appears to be one of reducing bacterial contamination of edible gelatin to a minimum through strict attention to plant sanitation. That some plants are much more successful than others in keeping down their counts is shown by the data in Table 6.

It has been suggested that the variable results we have obtained on replicate analysis of certain samples are due to the gelatin not being brought into complete solution. If this were true we would expect closer agreement between analyses of replicate 1-g. portions, which are more easily dissolved than are 11-g. portions. Actually, the opposite is true, as is clearly shown by the data in Tables 2 to 5. All our results point to the irregular distribution of bacteria in the powdered gelatin as the chief reason for variations between replicate samples. These variations, shown in the data in Tables 2 to 5, have persisted even when preliminary hydration and prolonged shaking were employed to obtain more complete solution. Consequently, it is difficult to understand how these would be overcome by taking a

smaller portion of gelatin (1 g. in place of 11 g.) for analysis. Nevertheless, every effort should be made to obtain complete solution of the gelatin. Rehydration by a preliminary period of soaking, together with the use of broken glass and mechanical shaking for 10 minutes after the gelatin appears to be dissolved, has consequently been adopted in our control work.

TABLE 6

Distribution of Bacteria Counts on Edible Gelatin from Selected Plants (July 29-December 6, 1949)

Plant	Total No. of samples	Count per gram				
		Less than 10,000	10,000 to 25,000	25,000 to 50,000	50,000 to 500,000	Over 500,000
		%	%	%	%	%
A	65	97.0	1.5	0.0	1.5	0.0
B	96	94.8	4.0	1.2	0.0	0.0
C	65	81.5	1.5	4.7	6.15	6.15
D	26	42.3	11.54	11.54	11.54	23.08

SUMMARY

The distribution of bacteria in powdered gelatin is highly irregular. Attempts to obtain a more uniform distribution by mechanical means were not encouraging. The sampling error may be reduced by examination of as large a portion (11 g.) as can conveniently be handled. This is considered to outweigh the advantages claimed for the use of the 1% solution (1 g. in 99 ml.).

Large variations in counts from replicate plates may reflect incomplete dissolving of the gelatin particles. A preliminary soaking to rehydrate the particles slightly reduced these variations and also resulted in better agreement between replicate analyses. Mechanical shaking with glass beads or broken glass after tempering at 45°C. to dissolve the particles was also of value. The incorporation of a wetting agent in the dilution water did not reduce the variability between replicate portions or replicate plates.

For plate counts, incubation temperatures of 32°C. and 37°C. for 48 hours were equally satisfactory. For coliform organisms, however, the lower temperature was definitely less satisfactory.

Acknowledgments

Grateful acknowledgment is made to Dr. A. H. Jones, Mr. J. G. Desmarais, and Miss B. Brawn for their help in connection with the analytical work, and to Dr. C. E. Chaplin for helpful suggestions in the planning and conduct of these investigations.

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